The Effects of Beta-hydroxybutyrate and Glucose on Breast Cancer Cell Cycle Regulation and Metabolism

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Abstract

Breast cancer is a leading cause of death among women in Canada. Obesity is a major modifiable risk factor for many cancers, including breast cancer. Thus, managing body fatness is looked upon as a potentially important intervention that can serve as an effective adjuvant therapy. Given this fact, the ketogenic diet (KD) has been garnering more attention as a novel cancer therapy due to its efficacy in inducing ketosis . The KD consists of high fat, low carbohydrate and moderate protein intake. KD consumption imparts a reduction in blood glucose, reduced insulin and increased ketone bodies. Cancers prioritize glucose as a fuel source for metabolism and often possess dysfunctional mitochondria. This results in a compromised ability to produce energy via oxidative phosphorylation and a high steady state ROS concentration. When isolating the above mentioned KD-dependent circulatory changes my results show minor adaptive cellular responses in MCF7 and MDA-MB-468 cells, with no effect on overall cell cycle status. Overall there appears to be a trend to shifting metabolism, however it still remains unclear as to the clinical potential of these changes. Thus, it is likely that the major benefit from KD is mediated by the weight loss and changes in adipokine secretion profile that accompany KD, rather than the reported alterations in blood insulin, glucaose and ketones in the circulation.

Acknowledgements

Firstly, I am deeply grateful to my research supervisor, Dr. Michael Connor. Thank you for your abundant support and guidance since I started in the Connor Lab in the midst of the pandemic. Your unwavering patience and expertise in this area has helped and inspired my research experience in the field of breast cancer. I would like to thank my lab mate, Ramy Habib for his counsel and instruction, for motivating me to persist in difficult situations and for the many laughs during frustrating moments.

This thesis is dedicated to my family: to my Father, Mother, and my little sisters; Chucky and Chippy. Thank you for your constant encouragement when I needed it the most, for the push to keep going when I felt like giving up, and for being my biggest cheerleaders, always cheering me on from the sidelines. Thank you for always praying for me. I hope I have made you proud.

Finally, and most importantly, I would like to thank God. Day after day He gave me the strength I needed to press on. In every moment, I was graciously reminded to look up and do all for the glory of the one who is the author and finisher of my faith.

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Abbreviations

AKT - Protein kinase B

AMPK - Adenosine monophosphate-activated protein kinase

AT- Adipose tissue

DMEM - Dulbecco’s modification of eagle’s medium

ATP - Adenosine triphosphate

BAT- Brown adipose tissue

bHB - Beta-hydroxybutyrate

BMI- Body mass idex

CAK - CDK activating kinase

CDK - Cyclin dependent kinase

CKI - Cyclin dependent kinase inhibitor

COX IV - Cytochrome c oxidase subunit 4

CVD- Cardiovascular disease

DNA - Deoxyribonucleic acid

ER+ - Estrogen receptor positive

ER- - Estrogen receptor negative

ETC- Electron transport chain

FFA- Free fatty acid

G0 - Gap 0 phase

G1 - Gap 1 phase

G2 - Gap 2 phase

GSH - Glutathione

INK4 - Inhibitor of kinase 4

KD - Ketogenic diet

KIP - Kinase inhibiting protein

M - Mitosis phase

mRNA - Messenger ribonucleic acid

mTOR - Mammalian target of rapamycin

OXPHOS- Oxidative phosphorylation

PI3K - Phosphatidylinositol 3-kinase

PR+ - Progesterone receptor positive

PR- - Progesterone receptor negative

PVDF - polyvinylidene difluoride

Rb - Retinoblastoma

ROS - Reactive oxygen species

S - Synthesis phase

TME- Tumour microenvironment

T2DM- Type 2 Diabetes Mellitus

WAT- White adiopose tissue

1.0 Overview of the Literature.

1.1 Cell cycle Overview

The cell cycle is a very complex and tightly regulated process that cells must undergo to achieve the end goal of cellular division into two identical daughter cells. When the cellular microenvironment favors growth, initiated by the presence of growth factors, the cell will enter the cell cycle, progressing through the different phases required for completion. The phases of the cell cycle consist of G0, G1 phase, S phase and M phase. In G1, the cell is metabolically active and is growing. The cell spends a significant amount of time in this phase increasing in cell size, and upregulating certain factors needed for DNA replication[1].

There are specific cell cycle checkpoints in place to establish that certain requirements have been fulfilled allowing the cell to progress to the next phase[2]. Three major checkpoints exist: the G1/S checkpoint, the G2/M DNA damage checkpoint, and the spindle assembly checkpoint[1]. The G1/S checkpoint, also known as the restriction point, occurs at the G1/S transition. Upon reaching the restriction point, the cell is then committed to progressing into S phase and through the rest of the cell cycle[2]. Prior to reaching this decision point, the cell will assess the environment and the growth conditions to determine whether it is plausible to support the cell division. Although crucial for initiation of cell cycle entry, growth factors are no longer required for the remaining of the cell cycle beyond the G1 restriction point as the cell is now committed[2]. Since transition through the cell cycle is a one-way process, it is crucial that the cell transitions from one phase to the next only when the previous phase has been fully completed[3].

To manage this proper sequential occurrence of phases, it is critical for the cell to strictly control cellular checkpoints. Cell cycle progression is largely regulated by proteins known as cyclins and cyclin dependent kinases (CDKs). CDKs are serine/threonine protein kinases which need to bind to their regulatory subunit proteins known as Cyclins. Together, this complex becomes activated and serves to function as one of the major control mechanisms during the transition between phases[4]. In order to maintain proper temporal sequence of events, catalytically active cyclin/CDK complexes are necessary to act at specific points in the cell cycle[4].

1.1.2 Cell Cycle Regulation.

Cyclin/CDK activities are regulated by multiple mechanisms including the transcription, synthesis & degradation of cyclins, site specific phosphorylation-dephosphorylation of CDKs, cyclin/CDK complex inhibitors and the control of subcellular localization of cyclins & CDKs[5] (Figure 1). CDK protein levels remain constant throughout the entirety of the cell cycle, while the temporal expression of the cyclins are dependent on the phase of the cell cycle that the cell is in[6]. Before entering the first active phase of the cell cycle, the cell remains in G0 phase, in a physiological state known as quiescence. This is a reversible, non-proliferative state where the cells are temporarily halted due to lack of the appropriate growth factors present in the microenvironment[7]. The presence of mitogenic signals in the environment promotes the assembling of the active Cyclin D-CDK4/6 complexes, containing a kinase inhibitor protein (Kip; p21/p27). In order for a cyclin D/CDK4 complex to be active, an initial intricate series of sequential events must occur. CDC25A is a phosphatase required to remove the inhibitory phosphorylation of Wee1 on tyrosine 15 on CDK4/6[8]. Cyclin activating kinase (CAK) must also phosphorylate CDK4/6 on threonine 172 for its activation[9]. Additionally, INK4 inhibitory proteins are bound to CKD4/6, thereby suppressing their activation by cyclin D[10]. These INK4 proteins include p16(INK4a), p15(INK4b), p18(INK4c), and p19(INK4d)[10]. p27^{KIP1} binds to the cyclin D-CDK4/6 complex displacing and removing the inhibition of the INK4 protein, allowing for an active cyclin D-CDK4/6 complex[11].

Upon receiving a mitogenic signal, the cell exits quiescence and upregulates the transcription of Cyclin D mRNA, resulting in an upregulation of Cyclin D protein. Cyclin D can then bind to its regulatory subunit, CDK 4/6. $p27^{KIP1}$ helps to assemble the cyclin D-CDK4-6 complex and allows for its activation. Once active, the cyclin D-CDK 4/6 complex can initiate the phosphorylation of its target protein, a tumour suppressor known as Retinoblastoma (Rb)[12]. The primary role of Rb is the inhibition of G1 phase cell cycle progression by its sequestration of E2F transcription factors[13]. The partial phosphorylation of Rb consequently results in its liberation of E2F, which is then able to begin transcription of numerous target genes, including cyclin E. $p27^{KIP1}$ is the primary inhibitor of the cyclin E/CDK2 complex. Therefore, the binding of $p27^{KIP1}$ to the Cyclin D-CDK4/6 complex that takes place in early G1 allows for its sequestration from its primary target cyclin E-CDK2. Active cyclin E-CDK2 complexes further phosphorylate Rb, resulting in a positive feedback loop of increased E2F activation and increased cyclin E mRNA/protein production. This results in the increase of cyclin E protein expression to levels above that of $p27^{KIP1}$ and other Kips, propelling the cell into S phase. Active cyclin E-CDK2 phosphorylates $p27^{KIP1}$ at T187, targeting the protein for degradation by SCF^{Skp2} ubiquitin ligase, which occurs in late G1 through S phase and into M phase[14]. The transition of the cell into S phase initiates DNA replication, with completion of DNA replication marking the end of this phase. Cyclin A is produced at the start of S phase, forming a complex with its subunit CDK2. Together, this active complex phosphorylates proteins that are vital to the DNA replication process[1]. Upon completion of S phase, Cyclin E is targeted for ubiquitin-mediated degradation by Fbw7 protein (F-box and WD repeat domain containing 7) as Cyclin A continues to increase. As cyclin A increases and binds to its respective cdk (cdk1/2), the cell progresses into G2 phase of the cell cycle[1]. Because G2 ends with the onset of mitosis, the major task within G2 is to ensure proper integrity of DNA replication in preparation for mitosis[1]. If mutations are detected, then the cell will be arrested in this phase to initiate DNA repair mechanisms. After completion of this checkpoint, the cell will prepare itself for entry into M phase for mitosis, by the activation of Cyclin B/cdk1 complexes. Because of the importance of its role as a mitotic inducer, the Cyclin B/cdk1 complex is tightly regulated[15]. After the synthesis of Cyclin B, inactive Cyclin B/cdk1 complexes are immediately activated by the Cdc25 and they phosphorylate substrates necessary for architectural reorganization for mitosis[15]. Mitosis consists of 5 distinct phases: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, chromosome condensation occurs[16]. During prometaphase, the spindle forms and attaches to the chromosomes reorganizing the cell into a double symmetrical like structure characteristic of metaphase[16]. When the metaphase chromatids have separated to opposite ends, telophase begins where the formation of 2 fully functional nuclei occurs in each of the 2 parts within the cell[16]. Subsequently cytokinesis occurs, during which a separation of the chromosomes and cytoplasm takes place resulting in two identical daughter cells[16].

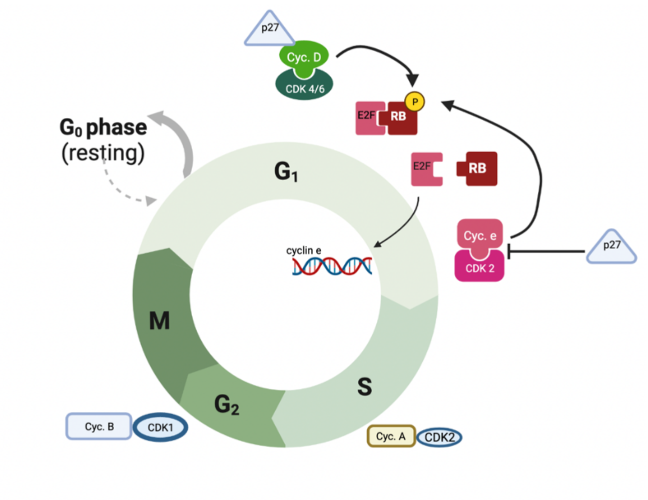

Figure 1: Cell cycle stages: A quiescent cell will remain in G0 phase until prompted by a mitogenic signal, after which the cell can enter G1 phase. Kip inhibitor p27 binds to the Cyclin D-CDK4/6 complex, activating it and sequestering it from its primary role of inhibition of target Cyclin E-CDK2. Cyclin D-CDK4/6 phosphorylates Retinoblastoma (Rb) dissociating it from its bound E2F transcription factors, which then enters the nucleus to upregulate the transcription of Cyclin E. Active Cyclin E-CDK2 levels above that of its inhibitor p27, allows for S-phase entry. Cyclin A-CDK2 regulates cell cycle entry into G2 phase, and Cyclin B-CDK1 is a mitotic inducer, regulating entry into M phase.

1.2 Cancer.

There are numerous genes that are responsible for the regulation of cellular processes. Cancer is driven by genetic mutations at the genomic level[17]. This disease is classified as a sporadic one in nature dependent on both genetic factors and environmental factors that influence DNA mutations and tumor formation[18]. Genes that determine tumor formation fall into 3 broad categories: oncogenes, tumor suppressor genes and DNA repair genes. A functional imbalance of oncogenes, tumor suppressor genes and DNA repair genes that favors proliferation and further mutations will lead to a progression of tumorigenesis[19]. As mutations accumulate, cancers transform demonstrating specific phenotypic characteristics including increases in tumor size, anti-apoptotic potential[20] and malignancy[19]. In 2000, *The Hallmarks of Cancer* were defined by authors Hanahan and Weinberg, categorizing a number of these distinguishing features including sustained proliferative signaling, evading growth suppressors, evading apoptosis, sustained angiogenesis, enabling replicative immortality, and activating invasion & metastasis[21] (Figure 2).

Subsequently, these hallmarks were updated adding reprogramming of cellular metabolism and avoidance of immune destruction, genome instability and tumor promoting inflammation to this list[22]. More extensive research within this decade has shed light on the significance of the tumor stroma in contributing to tumorigenesis and to the acquired distinguishing capabilities tumor cells obtain[23]. Being active contributors to tumor formation, the tumor stroma making up the tumor microenvironment is key in the onset, development and progression of the tumor[23]. Therefore, cancer research cannot any longer be limited solely to the biology of the tumor cells but must encompass the tumor microenvironment (TME) and its contributions to tumorigenesis.

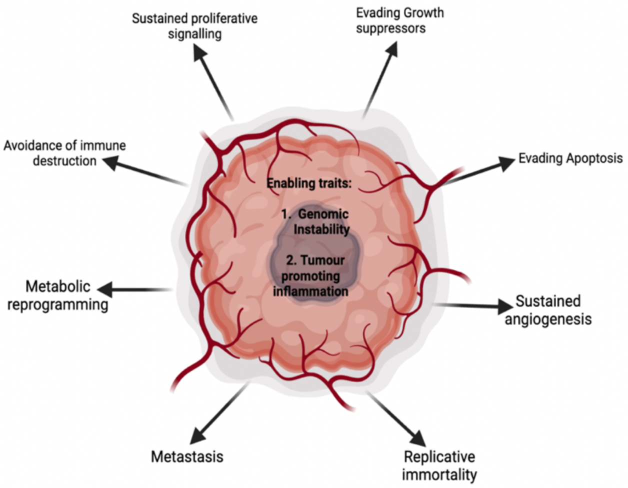

Figure 2: Hallmarks of Cancer: The hallmarks of cancer, as originally proposed by Weinberg and Hanahan, encompass 8 capabilities and 2 enabling traits of tumour cells during cell proliferation and tumor progression.

1.2.2 Metabolic reprogramming of Cancer cells.

One of the distinguishing factors that cancer cells acquire is metabolic reprogramming. Tumor cells have developed ways to adapt to changing metabolic demands of accelerated growth in order to survive. There are certain factors within the TME that induce metabolic reprogramming. The TME consists of a plethora of different cell types including immune cells, fibroblasts, endothelial cells and adipocytes, uniquely possessing different characteristics[24]. This can contribute to a difference in nutrient availability between non-tumor and tumor tissues. Additionally, tumors generally exist in hypoxic environments[24]. Due to insufficient oxygen supply, tumor cells undergo certain metabolic adaptations to be able to meet cellular energy demands[24]. Some of these adaptations include increased glycolysis and angiogenesis. Finally, another trait that characterizes the TME is extracellular acidity as glycolytic tumors often produce an excess of lactic acid. Taken together, these factors all influence the metabolic flexibility of cancer cells (Figure 3).

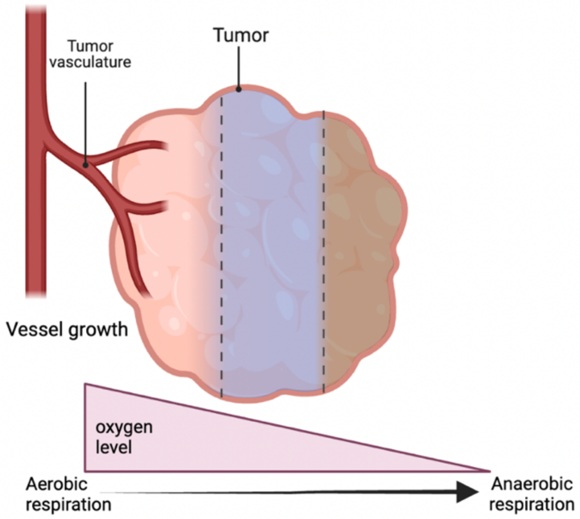

Figure 3: Hypoxic environment of tumor cells: As tumor cell proliferation progresses, there is a reduction in oxygen supply to the tissue leading to a hypoxic environment. The under vascularization of the tumor promotes an increase in glycolytic metabolism.

The quintessential cell intrinsic metabolically reprogrammed pathway is termed aerobic glycolysis and was discovered in the 1920s by German scientist Otto Warburg[25]. Non-cancerous cells produce most of their energy through aerobic respiration utilizing the mitochondria, as it is the more efficient mechanism[26]. Upon oxidation of glucose, the resultant pyruvate can then enter the mitochondria and cycle through the Krebs cycle to be fully oxidized, from which a net of 4 molecules of Adenosine Triphosphate (ATP) are yielded[27]. The NADH and FADH produced from glycolysis and Krebs cycle can then be shuttled to the mitochondria for use in the electron transport chain, which will ultimately result in rapid production of 36 ATP. Dr. Otto Warburg discovered that cancer cells favoured glycolysis/fermentation as the main energy producing pathway, observed by the increase in lactate production, even in normoxic conditions[28]. This shift in metabolic preference is known as the “Warburg effect” (Figure 4). Metabolic reprogramming allows for tumour cells to support catabolism during conditions of nutrient and oxygen deficiency through the preferential uptake of glucose, and to a lesser extent amino acids[29]. This is important because tumors are “under vascularized’ compared to other non-tumor tissues. Although the Warburg phenotype has been reported in many tumour types, some cancers exist that preferentially undergo oxidative phosphorylation (OXPHOS) in glucose limiting conditions[29]. This is known as metabolic flexibility whereby cancer cells can adapt metabolically in unfavourable conditions (i.e. low glucose) to selectively favour their growth[29].

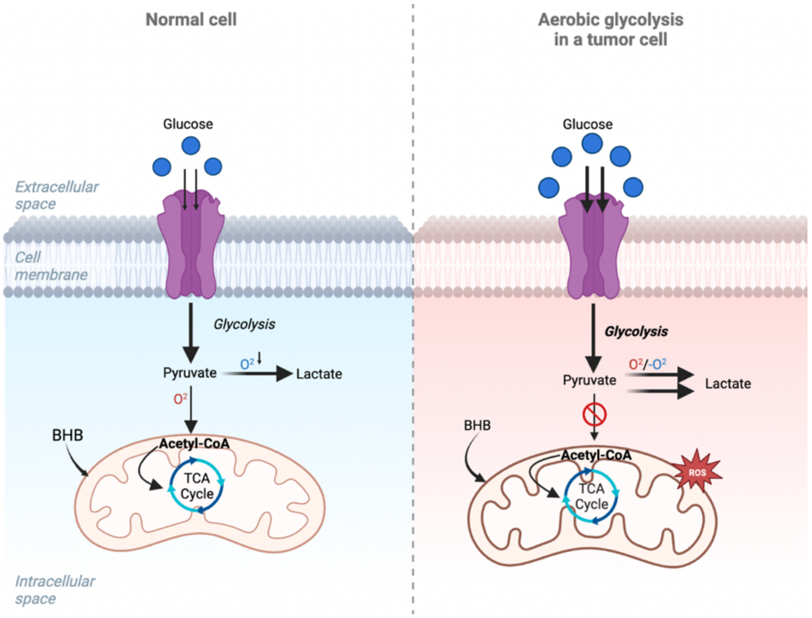

Figure 4: The Warburg Effect: In a normal cell, the metabolism of the cells are determined by the availability of oxygen and the substrate. In the presence of oxygen, glucose is catabolized into pyruvate via the process of glycolysis. Pyruvate can then enter the mitochondrion where it is first converted into Acetyl Co-A, the first step in the process of aerobic respiration. In anaerobic conditions, pyruvate is converted into lactate, a process known as lactic acid fermentation. In tumor cells across many tumors cell lines, aerobic glycolysis has been observed where glycolysis is heavily relied upon as a primary energy source, and a lack of

aerobic respiration. Due to its primary reliance on glycolysis, a heavy glucose intake is required to support the metabolic demands of the cell.

1.2.3 Treatment.

According to the Canadian cancer society, it is estimated that 43% of Canadians will receive a cancer diagnosis in their lifetime. Although incidence and mortality rates of breast cancer amongst females are decreasing, mortality rate is 19%, which actually represents an increasing number of deaths within the population given the increased incidence of cancer among Canadians[30]. Even within breast cancer, different types have different origins and phenotypes, thus having divergent biological & pathological features, clinical manifestations, and responses to therapy[31]. For this reason, treatment must be specially tailored to the type of cancer and obtain tumour specificity, since there are wide variations in genetic alterations within the same cancer type. Traditionally, cancer treatments have been focused on impacting the neoplastic cells[32]. Current practices include surgery, radiation, chemotherapy, and immunotherapy[32]. Early stage and localization of the cancer is when surgery can be most effective. The main objective of surgery is the physical removal of the tumor mass or cancerous tissue. In cases where all of the tumor cannot be removed, cytoreductive surgery may be performed, which is the surgical removal of as much of the tumor mass as possible combined with chemotherapy or radiation, making the 2 latter treatments more effective. Chemotherapy involves the administration of anticancer drugs that inhibit the growth of and/or kill the cancer cells[33]. The concoction of drugs administered are dependent upon the type of cancer, and the location[33]. Although the use of chemotherapy is wide spread and it is proven to be an effective method of treatment, many severe side effects related to the destruction of healthy tissue are a consequential reality. Commonly used in conjunction with surgery is radiation therapy. Radiation therapy uses high energy ionizing radiation to destroy the tumor cells by targeting & damaging DNA directly, or by producing free radicals which then target the DNA[33]. Over the past few decades, tremendous progress has been made in the field of immunotherapy, the method by which the immune system of the patient targets the tumor cells to control and eliminate them. The objective of immunotherapy is to upgrade the immune defence system of the patient to recognize, and selectively target neoplastic cells, proving to be advantageous as the healthy cells are not being destroyed in the process[34]. Immunotherapy is relatively low risk with minimal side effects, presenting itself as a safe option for therapy. Despite the promising potential, ,immunotherapy still remains in its infancy.

1.2.4 Cancer and ROS.

Reactive Oxygen Species (ROS) is a collective term to describe molecules derived from the partial reduction of oxygen. Examples of ROS include Hydrogen peroxide (H_2O_2), Superoxide ($O2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) molecules[35]. These free radicals have an unpaired electron on their outer shell, making them highly reactive, collectively making them a cause for potential damage to the cell. ROS homeostasis is vital in the functioning and signalling of the cell, with a dynamic balance maintained by reduction-oxidation reactions and sufficient antioxidant activity[36]. The basic survival of cells is dependent on sustained homeostatic physiological ROS levels[36]. However, abnormalities in ROS concentrations during the onset and progression of cancer can exert paradoxical effects on the proliferative capability and the death of cells[37]. Increased ROS concentrations lead to a mild oxidative state which has been found to promote tumorigenesis. This is described as a hormetic effect, which is where increased exposure to a specific stressor can be beneficial to the growth of the cancer cells below a certain threshold[36]. In contrast, excessive exposure to ROS can disrupt metabolic and cellular processes, consequently promoting a toxic environment for the cells leading to cell death or growth arrest[38] (Figure 5). A feature of cancer cells is that they exist in a state of persistent oxidative stress, a result of ROS overproduction and inactivation of sufficient antioxidant pathways[38]. One of the primary sources responsible for ROS generation is the mitochondrial electron transport chain (ETC)[39]. Mitochondria are vital organelles involved in the production of energy, cellular metabolism and apoptosis[40]. During oxidative phosphorylation, electron ‘slip’ occurs, whereby some electrons can leak from the ETC as they pass through. Approximately 1% of molecular oxygen reacts with these ‘leaked’ electrons, yielding superoxide, most of which is dismutated to Hydrogen Peroxide (H_2O_2)[37]. The mitochondria of numerous types of cancer cells have been widely known to be of comprised function, due to structural impairments[41]. Defects in mitochondrial DNA (mtDNA) have been observed in the mitochondria of cancer tissues including breast, thyroid, colon, neck and prostate cancers[42] supporting evidence of the dysfunctionality of the mitochondria in cancer cells[42]. These functional impairments make tumor cells susceptible to overproduction of mitochondrial ROS (mtROS). The increase in mtROS may also contribute to further induction of mitochondrial DNA alterations, which can induce apoptosis[43]. Therefore, the homeostatic balance between the levels of ROS and the regulation of antioxidant mechanisms is critical in the survival of tumour cells[38]. Thus, elevated ROS levels may be a means to induce apoptosis or other forms of cell death in cancer cells.

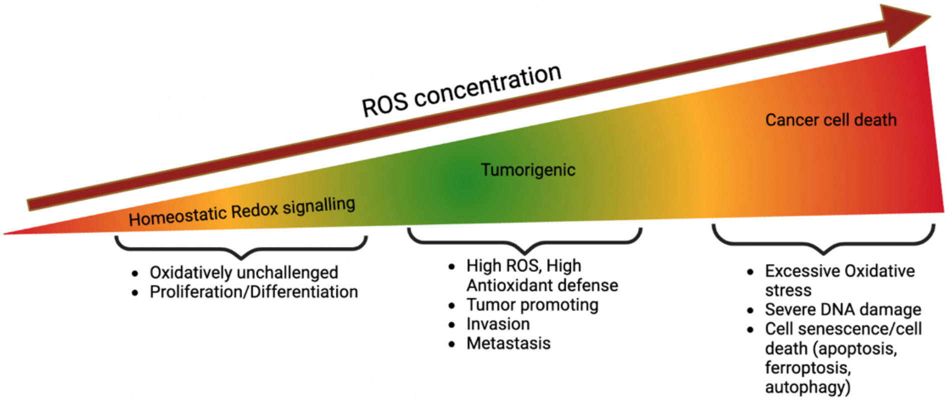

Figure 5: ROS and Cancer paradox: ROS homeostasis is vital to the functioning of cells. When the concentration of ROS is within homeostatic range, there is a balance of ROS and Antioxidant defenses which is adequate to promote proliferation. A further increase in ROS is met with high antioxidant defense to meet the oxidative demands of the cell. This oxidatively challenged state is favorable for tumor cells, promoting tumor initiation & progression, invasion, and metastasis. An excessive oxidative state stems from an imbalance of ROS and antioxidant defenses, promoting severe DNA damage and cell senescence and/or cell death.

1.3 Obesity.

Obesity once affected primarily the most affluent societies but now represents a third of the world's adult population[44]. Over the past two decades, the obesity epidemic has been growing on a global scale with increases in prevalence in most countries since the 1980s[45]. From 1975 to 2016, its worldwide incidence has tripled[46] with (according to the Canadian community health survey) approximately 1 in 4 Canadians living with obesity. Although body mass index (BMI) has been found to be an unreliable measure of obesity, it is still the standard measure worldwide. A person is considered to be obese when body mass index (BMI) is over 30 kg/m^{2}on a BMI scale, according to the World health organization (WHO)[47.] The causes underlying the explosion of this epidemic are multifactorial, with lifestyle factors playing a dominant role in the development of this disease. These include certain environmental, behavioural, and genetic factors such as low socio-economic status (junk food is more affordable & accessible in western society), sedentary lifestyle and high availability of high caloric density foods[47]. Simply put, obesity is excessive fat accumulation that presents an increased risk to the health of the individual and it develops in part, when caloric intake significantly exceeds energy expenditure[47]. Obesity is a significant risk factor for many chronic diseases such as cardiovascular disease (CVD), type 2 diabetes, cancer, osteoarthritis, liver & kidney disease, sleep apnea and depression[48]. Treating these conditions places a heavy weight on the healthcare system[49]. Obese individuals are estimated to have a 30% higher medical cost than individuals with a lower BMI[49]. As well, it is strongly linked with an increased risk of all-cause mortality, cardiovascular mortality, and cancer mortality[48].

Pathogenesis of Obesity on a Molecular level: Adipose tissue.

Underlying the complications of obesity is dysfunction at the molecular level that throws the body out of its homeostatic state. To clearly understand the etiology of obesity, it is imperative to start by recognizing the importance and contributions of adipose tissue to homeostasis. Adipose tissue (AT) is a dynamic organ found throughout the body that functions as the main energy storage depot[50]. Adipose tissue can be further classified as either white adipose tissue (WAT) and brown adipose tissue (BAT)[51]. Morphologically WAT consists of large adipocytes (fat cells) with a unilocular lipid droplet and few mitochondria, while BAT consists of multilocular lipid droplets and many mitochondria[51]. Therefore, the function of WAT is energy storage and maintaining a homeostatic balance in response to caloric imbalance[51].

During the process of digestion, complex carbohydrates and triglycerides are broken down into their absorbable units; monosaccharides and monoglycerides & free fatty acids[52]. Due to limited storage capacity, glycogen is insufficient to support metabolic needs under prolonged fasting conditions, being able to support less than a day's worth of energy demand[52]. Additional circulating glucose is then converted and used to synthesize triglycerides. During a prolonged period of fasting, the primary metabolic fuel is obtained from the breakdown of triglycerides[52]. into glycerol and fatty acids. Fatty acids can be used by the cell as a fuel source either via β-oxidation (direct) or the conversion of fatty acids by the liver into acids known as ketone bodies (indirect)[52]. Not only does WAT acts as an energy storage reservoir, but it is also responsible for the secretion of paracrine factors that are important for the regulation of other metabolic tissues[53]. In caloric excess, AT responds via adipocyte expansion. Healthy adipocyte expansion occurs through adipocyte hyperplasia,an increase in adipocyte number in response to energy excess, that has protective effects against the metabolic complications of obesity[54]. In obese conditions (extreme caloric surplus), adipocytes adapt via adipocyte hypertrophy- an expansion of adipocytes[54]. This unhealthy adipocyte expansion promotes a multitude of detrimental effects including inflammation, hypoxia, and altered adipokine secretion[55] and the obesity associated metabolic conditions.

1.3.2 Carbohydrate Intake and Obesity.

Western society has propelled itself towards providing an “obesogenic” environment for its population, with the increased marketing of sugar dense foods & beverages, and the increased availability of high fat high sugar “junk” foods[55]. There currently exist 2 primary paradigms under which scientists are attempting to explain the causal factor underlying the obesity epidemic. It is still the most widely held belief that the disease of obesity can be conceptualized using a “calories-in calories-out” model, where obesity is defined as merely a disorder of energy balance[56]. However, adopting this approach is appropriating a mere principle of physics, without much consideration of the many complex biological mechanisms that are involved[57]. More recently, it has been shown that, in animals, diet composition does show an effect on body composition and metabolism independent of caloric intake[56]. Excessive sugar consumption is positively associated with an increased risk of developing obesity[58]. Moreover, excessive sugar consumption may also promote the development of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM), diseases that are part of the metabolic syndrome which accompanies obesity. There are multiple plausible mechanisms suggesting indirect and direct negative effects of sugar on the progression of these diseases. Evidence suggests that fructose increase can directly cause dysregulation of lipid and carbohydrate metabolism, while also indirectly causing metabolic dysregulation due to the effects of sugar consumption on positive energy balance and fat gain[59]. Several epidemiological studies have shown a significant positive relationship between sweetened beverage consumption and BMI, even during isocaloric conditions[60].

Intervention studies have shown that consumption of *ad libitum* high sugar diets promoted body weight gain, whereas consumption of *ad libitum* low sugar diets showed no increase in body weight[59]. Taken together, the effects of sugar, independent of adiposity, may perhaps explain the correlational relationship with the steady increase in obesity related disorders, and obesity itself.

1.4 Obesity & Cancer.

As more deleterious activities are being taken up by western society including the increased intake of sugar dense processed foods & the increase in sedentary activity, more studies have looked at the associations between these and cancer. Traditionally, cancer treatments have focused on the neoplastic cells themselves with chemotherapy, surgery and radiation targeting the proliferating cancer cells. However, TME characterizes the non-cancerous cells surrounding the tumor, and it may have predictive significance for the behaviour of the tumor itself[32]. Most cancers are primarily surrounded by blood vessels, adipocytes, stromal cells, and immune cells, often a part of the TME. Adipose tissue serves as an endocrine/paracrine organ, secreting hormones which may act on neighbouring cells and/or enter the systemic circulation which have been shown to play a role in the direct link between adipose and tumour growth[61]. For over 50 years, the link between obesity and breast cancer has been known[61]. In a study conducted by Chang et al., it was found that a high BMI was significantly associated with increased risk of developing inflammatory breast cancer (IBC), a very aggressive class of cancer in both pre-menopausal and post-menopausal women[62]. The most abundant adipokines produced and secreted by the adipose tissue are Leptin and Adiponectin. Adiponectin has been found to be negatively correlated with obesity, type 2 diabetes and insulin resistance in both humans & rodents, and high levels of serum adiponectin are associated with anti-inflammatory effects[63].

Leptin is a regulator of body weight and energy intake. Several epidemiological studies have shown an increase in serum leptin levels in breast cancer patients when compared to healthy subjects. As well, leptin was found to be overexpressed in breast cancer tissue biopsies compared to non-cancer breast epithelium. In addition to the implications that these hormones may have in adiposity, Leptin and Adiponectin also affect cell cycle regulation in breast cancer cells[64]. Previous work in our lab has shown an increase in MCF7 cell proliferation after being grown in conditioned media created from the adipose tissue of "obese" animals fed a high fat diet, as compared to cells that were treated with conditioned media created from the adipose tissue of "lean" chow diet fed animals[65]. Given the growing evidence for the strong role that adiposity plays on cancer cell cycle regulation, it is imperative to continue to investigate ways in which adiposity can be manipulated to achieve a beneficial adipokine ratio, as it relates to tumour progression. Therefore, finding targeted cancer treatments are vital in changing the cancer paradigm and can improve success in the search for effective treatments.

1.4 Ketogenic Diet.

In 1911, a pair of Parisian physicians formally carried out a fasting intervention in epilepsy patients and concluded that seizures were less severe in the duration of the treatment[66]. In 1921, the culmination of years of fasting treatments & interventions finally reached a pivotal point that led to two significant conclusions. It was noted by Woody-att, that a fasting state induced the production of circulating acetone and beta-hydroxybutyric acid (βHB) in normal subjects on a diet that contains a significantly low proportion of carbohydrates[67]. Secondly, Wilders put forth the idea that the sole benefits of fasting could be obtained at the onset of ketonemia[68]. He then proposed that any ketone producing diet could possibly relieve epileptic patients of their seizures. Hence, Wilder coined the term “ketogenic” diet, and this form of intervention has been implemented ever since[66]. Unfortunately, the KD did not continue to receive attention, with only two to eight publications per year on PubMed from 1970-2000[66]. However, the KD has experienced a re-emergence in the last 2 decades after the airing of an NBC program special on the true story of a two-year-old boy who was completely healed of his seizures since being on a KD[66]. PubMed saw a dramatic increase of over 40 publications a year since then[66]. Essentially, this nutritional intervention consists of no more than 50 g/day in carbohydrates, moderate protein intake and majority of caloric energy from fat. The carbohydrate restriction results in lower blood glucose levels and lower insulin levels. Decreased circulating insulin consequently leads to a sharp decrease in fat and glucose storage, promoting lipolysis and the release of free fatty acids (FFA) from adipose tissue[69]. FFAs then enter the liver where they are metabolized via beta oxidation into acetoacetate which is subsequently converted into βHB and acetone, compounds known as ketone bodies[70]. This process is known as ketogenesis and it marks the body’s switch into a metabolic state known as ketosis, whereby blood ketone levels are elevated. During this process, three major ketone bodies are formed: acetone, acetoacetate and βHB, of which the latter is the primary circulating ketone body. βHB reaches physiological plasma levels of 2-6 mM while in this state of ketosis. These ketone bodies can then enter the circulation where they are subsequently taken up into other tissues including the brain, to be used as an alternative source of energy[71]. For the tissues to use βHB as a source of energy, it needs to be converted to acetyl-CoA, which can then be used in the Krebs cycle[71]. During physiological ketosis, blood ketone body levels typically reach maximum levels of 7/8 mmol/L[72].

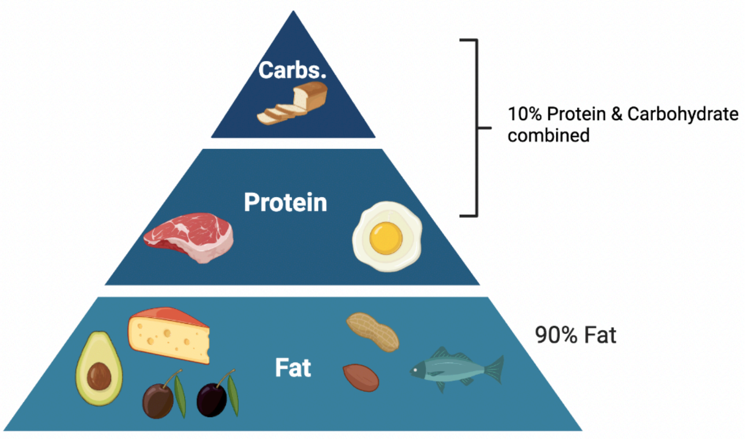

Figure 6: The Ketogenic diet pyramid: The ketogenic diet consists of a high fat intake, with approx. 90% of the daily caloric needs being met through fat, and a low carbohydrate intake with 10% being met through combined protein and carbohydrate intake.

1.4.2 Ketogenic Diet & Obesity.

Considering the overwhelming increase in obesity trends, different interventions need to be evaluated to ameliorate the trajectory of this epidemic, for the well-being of both the individual and society. Strong supportive evidence suggests that the KD is effective for weight loss although the exact underlying mechanisms remain unknown[72]. Historically, low fat diets have been advised for weight loss and improving obesity related comorbidities based on the correlational relationship between dietary fat intake and cardiovascular risk[73]. However, multiple lines of evidence have shown that low fat diets may not be the most effective for weight management. A Cochrane collaboration review was published over a decade ago suggesting there were no clinical benefits of a low-fat diet when looking at its association with weight loss, compared to caloric restriction[74]. As well, clinical trial evidence from 1983 that was responsible for influencing dietary guidelines in the U.S and the U.K at the time, failed to demonstrate a favoured outcome of low-fat diets for those classified as overweight or obese[75]. Sackner-Bernstein et al. conducted a meta-analysis of randomized controlled trials, comparing low carbohydrate diets (LoCHO) with low fat diets (LoFAT) as a dietary intervention for overweight/obese adults and assessed the efficacy of various parameters including weight and cardiovascular disease (CVD) risk[73.] This meta-analysis found that in strict adherent populations, the LoCHO diet was associated with significantly greater improvements in weight loss when compared to the LoFAT diet from 8 weeks to 24 months of duration[73.] Aside from reduced weight loss that is seen to occur with adherence to the KD, there are other obesity related beneficial effects that have been observed. Obesity is often accompanied by the development of other comorbidities such as type 2 diabetes, cardiovascular diseases, and cancer[76]. As such, the KD has also been found to exert positive effects on overweight/obese individuals in addition to the fat and weight loss. Within the first 6-12 months of initiating the KD, transient decreases in blood pressure and increases in high density lipoprotein (HDL) have been demonstrated[77]. Atherogenic dyslipidemia, a disease characterized by low HDL cholesterol levels, is very prevalent among the obese population[78] making the HDL increasing effects of the KD of benefit to patients. Insulin resistance characterizes type 2 diabetes mellitus and is seen commonly in the obese population. The KD has been shown to increase insulin sensitivity and improve glycaemic control[72]. Current research suggests that a ketogenic diet is more effective in improving obesity related parameters when compared to a low-fat diet, challenging the guidelines that have been adopted in western society over the past few decades. This position necessitates further research into the efficacy and limitations of implementing such an intervention.

1.4.3 Ketogenic diet and Cancer.

Although there have been vast advancements in cancer therapeutics, traditional anti-cancer therapy still presents various setbacks including therapy resistance, lack of response and adverse side effects[79]. Numerous preclinical studies have demonstrated evidence for an anti-tumor effect of the KD in multiple cancer cell lines[80]. Many cancer cells have a predominately glycolytic phenotype with the Warburg effect responsible for satisfying the metabolic demands of the cells[81]. Because the KD is associated with a decrease in blood glucose, it has been studied as a therapeutic means to exploit the metabolic reprogramming of cancer cells, causing a forced shift in substrate utilization[81]. As well, many cancers/cell lines also lack functional mitochondria, with either certain compromised mitochondrial DNA mutations and/or defects in mitochondrial enzymes[82]. Due to this dysfunction and the persistent oxidative stress that cancer cells exist in, some cancer subtypes lack the ability to effectively metabolize ketone bodies[80]. Prior research has primarily focused on a combined approach, looking at the efficacy of the ketogenic diet in combination with standard cancer therapy, to investigate the effects on quality of life, strengthening the efficacy of conventional chemotherapy and overall safety[83]. Previous unpublished research in our lab has shown a downregulation of the AKT signalling pathway responsible for cellular survival in MCF7 cells, in response to glucose depleted conditions. Additionally, an upregulation of the energy sensing protein AMPK was found, in response to βHB in low glucose environment. In addition to the effects of decreased circulating insulin in downregulating the activation of the signalling mTOR/AKT pathway, it has been shown in mice that a KD upregulates AMPK in mice, inhibiting the mTOR/AKT pathway[72], confirming the results our lab has found *in vitro*. As well, class 1 histone deacetylases (HDACs), a family of proteins primarily responsible for supressing gene expression by deacetylating lysine residues on histone proteins, may be inhibited byβHB leading to aninhibition of tumor growth[72]. In addition to the molecular benefits of the KD in improving cancer outcomes, the adipose-specific benefits increase2-fold when the KD is adhered to. Decreased adiposity has dramatic effects on cancer cell growth. However, it is still unclear whether the physiological effects of the KD may have cancer cell cycle implications or whether these effects are due to primarily to weight loss and the associated alterations in adipokine secretion profiles that contribute to the TME. Furthermore, it is very difficult to separate the KD-associated ciruculatory effects from the adiposity related effects *in vivo*. Based on multiple lines of evidence, the KD has been supported as an adjuvant breast cancer therapy in preclinical and some clinical studies, therefore the underlying physiology specific mechanisms should be investigated[84].

2.0- Research Objectives:

As reflected in the existing literature, the physiological changes induced by the ketogenic diet namely decreased glucose & insulin and increased ketone bodies, may elicit metabolic effects pertaining to cancer cells. These physiological changes may drive the cells to switch their metabolic preference from glycolysis to oxidative phosphorylation. As well, because cancer cells often have compromised mitochondrial integrity and exist in a hypoxic environment, ketogenic specific physiological changes may force an increase in oxidative stress, potentially leading to a more pronounced increase in ROS production, making the cancer cells susceptible to cell death/cell cycle arrest. This study will isolate the circulatory "benefits" of the ketogenic diet and investigate their effects on cancer cells, without the influence of reduced adiposity and adipokine ratio on cell proliferation.

2.1- Hypothesis:

1. A low glucose/high βHB environment will facilitate a slowing of growth in breast cancer cells
2. The effects of βHB and glucose changes seen in ketosis will be also affect cell growth in a more advanced/aggressive breast cancer cell line.
3. A low glucose/high βHB environment will counteract the Warburg shift and increase mitochondrial ROS emission.

3.0- Manuscript:

The Effects of Beta-hydroxybutyrate and Glucose on Breast Cancer Cell Cycle Regulation and Metabolism

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3.1- Abstract:

The ketogenic diet (KD) has historically been a dietary intervention for epileptic seizures and more recently fat loss. KD elicits physiological effects which primarily include a decrease in glucose,a decrease in insulin and an increase in ketone bodies. The KD has been a proposed intervention for cancer prevention and treatment due to the glycolytic metabolic preference of most cancer cells, and their altered metabolic properties. This study had 2 aims: 1) to investigate the effects of manipulating glucose, insulin and Beta-hydroxybutyrate (βHB) levels to mimic physiological ketosis on breast cancer cell proliferation and 2) to investigate a potential role of ROS in mediating the cell cycle effects. Our results phenotypic changes on protein expression with no overall effect on cell cycle status. There was an increase in activation of the energy sensing protein AMPK after 48 hrs in both MCF7 and MDA-MB-468 cell lines in glucose reduced conditions. A parallel reduction in total AKT was also observed MDA-MB-468 cells, pointing to an inhibitory effect of AMPK on AKT. Oxidative stress, as measured by observing extracellular H_20_2 concentration, was increased in the glucose depleted groups with high

concentration of BHB (7mM) in both cell lines. These findings suggest that although serological changes of the KD may induce metabolic effects, there are no negative growth effects.

3.2- Introduction:

Cancer is the leading cause of mortality in Canada, accounting for 28% of all deaths[30]. Although advances have been made to curb the trajectory of this disease, many drawbacks exist with the current treatments available. Current cancer therapeutics include chemotherapy, radiation therapy, surgery, and immunotherapy. Although these interventions have proven to be successful in affecting tumor growth, there are many adverse systemic side effects associated with these strategies, drastically decreasing treatment success and patient quality of life. The focus of the interventions currently available today have largely targeted the proliferating neoplastic cells themselves, however the effects of these therapies are often not tumor-specific. Furthermore, the adaptive nature of tumors make them difficult to effectively treat long-term. In contrast, the tumour microenvironment (TME) is a stable component of patient physiology and has been found to be a predictive indicator of tumor behaviour/growth potential[32]. There is a dynamic relationship between the tumor and the TME that allows for the survival and growth of the tumor itself[32]. Most tumors are surrounded by fat tissue, which is comprised of immune cells, stromal cells and adipocytes, all of which produce and secrete hormones that can elicit growth effects on tumors in a paracrine/endocrine manner[61]. Thus, the TME represents a novel area of investigation in cancer research. To this, our lab has previously observed an increase in MCF7 cell proliferation after being exposed to conditioned media created from the adipose tissue of animals fed a high fat diet, relative to cells that were treated with conditioned media created from the adipose tissue of animals fed a lean chow diet[65]. This points directly to the notion that factors produced and/or secreted by the tissues/cells surrounding tumors controlling cell growth.

Over the years many different strategies have been directed at weight loss. The ketogenic diet, historically used as a therapeutic measure for epileptic seizures, is one of these strategies and has been proven to be efficacious for fat loss and obesity management[85]. Nutritional ketosis is attained through the restriction of carbohydrate intake, moderate protein intake and increased fat consumption[70]. Because glucose is the body's primary metabolic fuel, there is a shift in substrate preference during ketosis from glucose that occurs because of decreased carbohydrate intake and availability[89]. Fat metabolism occurring in the liver is used as a substrate to produce ketone bodies[68] (Acetyl coA, beta hydroxybutyrate, and acetone). This leads to an increase in the circulation of these ketone bodies, with Beta hydroxybutyrate (βHB) being the most abundant in the circulation. These ketones can then be used as a metabolic source by extra-hepatic tissue[85]. The efficacy of the KD in adipose reduction also presents implications for breast cancer as a result of an altered adipokine profile ratioto one that does not favour breast cancer proliferation.

Aside from the primary adiposity related cancer cell cycle effects that can occur, KD consumption also results in decreased glucose, decreased insulin, and increased ketone bodies[70], each of which could potentially alter the make up of the TME and elicit growth inhibitory effects on tumor cells. Many cancer cells have altered metabolic properties, whereby they preferentially metabolize glucose compared to non-cancer cells, even in aerobic conditions, to support their growth and survival[40]. This phenomenon is known as the Warburg effect, named after Otto

Warburg, who directly observed that tumour cells were heavily converting pyruvate into lactic acid to produce ATP regardless of oxygen status[25]. The primary reliance on anaerobic metabolic pathways allows the cell to survive and proliferate in hypoxic conditions, as many tumours are under vascularized and exist in a hypoxic environment[20]. Furthermore, many tumour cells also have mitochondrial dysfunction and this Warburg shift allows for cellular energy demands under these conditions to be met. Due to the drastic decrease in carbohydrate intake experienced with adherence to the KD, there is a decrease in blood glucose and insulin. Thus, decreasing blood glucose may be a means of exploiting this altered metabolism to potentially force a reversal of the Warburg effect. This may be beneficial by forcing tumor cells to utilize oxidative phosphorylation which may further increase ROS production and concentration, resulting in potential cell cycle arrest or even apoptosis.

3.3- Methods.

Cell Culture:

MCF7 breast cancer cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Burlington, ON). The DMEM was supplemented with 5% FBS (Wisent, Montreal), 3% antibiotic antimycotic (Wisent), 1% sodium pyruvate (Sigma, Missisauga ON) , 1% non-essential amino acids (Sigma), and 10 µg/ml of insulin (Sigma). MDA-MB-468 breast cancer cells were grown in DMEM with 10% FBS (Wisent), 1% antibiotic antimycotic (Wisent), 1% sodium pyruvate, and 1% non-essential amino acids (Sigma). All cells were maintained and cultured in T75 Falcon flasks (Fisher Scientific, Whitby, ON) incubated at 37°C and 5% CO_2.

Treatment:

MCF7 and MDA-MB-468 breast cancer cells were plated in 6 well plates (Sarstedt, Montreal, QC). 24 hours post-seeding, cells were exposed to vaired glucose concentrations ranging from 2-10 mM. In addition, a subset of cells were exposed to βHB (Sigma) at a concentration of 7 mM in both 2.5 mM and 5 mM glucose environments. Cells were exposed to treatment for 48 hours or 72 hours before harvesting.

Cell Collection:

After treatment, media was aspirated and cells were rinsed twice with ice-cold Phosphate Buffer Saline (PBS, Wisent). Rinsed cells were scraped from the wells and transferred to 1.7 mL microcentrifuge tubes and centrifuged at 2,320 x g at 4°C for 10 minutes. Following centrifugation, the supernatant was removed, and the pellet was resuspended in TENT buffer (0.2% TENT- TRIS, EDTA, NaCl, 0.2% Triton x-100) and 1% phosphatase inhibitor (PHOSstop, Roche, Montreal, QC) and protease inhibitor (Sigma). Cells were then sonicated for 3 seconds on ice. Subsequently, cells were centrifuged at 16,168 x g at 4°C for 10 minutes. The supernatant was collected and stored at -80°C for future analyses.

Immunoblotting:

Bradford assays were conducted to determine protein concentrations of cell lysates. 25 μg of protein were subjected to standard Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% and 15% gels). Following separation, proteins were then transferred to a PVDF membrane (Bio-rad, Missisauga, ON) overnight at 40V (4°C). Upon transfer, amido black staining was used to assess transfer efficiency. Membranes were then blocked in 10% milk for 2 hours at room temperature. They were then washed with TBST three times and incubated overnight with primary antibodies in 5% bovine serum albumin at 4°C with agitation. Antibodies of interest included AMPK (Cell Signalling), pAMPK^{T172} (Cell Signalling), AKT (Cell Signalling), pAKT^{T308} (Cell Signalling), Cyclin E (Abcam, MA), p27 (BD Biosciences, Missisauga, ON), COX4, and β-actin (Abcam). Membranes were then washed and incubated with appropriate specific HRP-linked secondary antibodies in 5% milk at room temperature with shaking for one hour. Membranes were washed three times with TBST and protein bands were visualized using enhanced chemiluminescence substrates (immobilon, Millipore) with a Kodak

In Vivo FX Pro imager. Images were quantified using Carestream software (Carestream, Rochester, NY) with β-actin used as a loading control.

Cellular H202 Emission Measurement:

To assess ROS secretion we employed an amplex red capture protocol modified from one described previously[86] . MCF7 and MDA-MB-468 cells were plated on 6-well cell culture plates. Following 72 hours of treatment 10 μM Amplex UltraRed and 1 U/mL horseradish peroxidase was added to each well for 60 min and fluorescence (EX568/EM581) was measured using the BioTek Synergy HTX fluorescent plate reader (BioTek, Winooski, VT). Readings reflected the net accumulated fluorescent resorufin product of oxidized Amplex Ultrared and represents the "net emission" of H_2O_2 that accumulated of the 60 minutes, representing a "live capture" of H_2O_2 release. Data were normalized to a H_2O_2 standard curve and protein content was determined by standard Bradford protein assays. H_2O_2 emission was expressed as absolute values and values corrected for total protein content.

Cell Cycle Analyses:

Treated MCF7 and MDA-MB-468 cells were collected by trypsinization, washed in PBS twice, and fixed by drop wise addition of ice-cold 70% ethanol. Prior to analyses fixed cells were washed in PBS and resuspended in a propidium iodide/RNAse solution overnight, and FACS analyses were run the next day with Attune NxT flow cytometer. Mod-fit software (Verity Software House, Topsham, ME) was used to determine cell cycle profiles.

Statistical Analysis

Statistical analyses were conducted GraphPad Prism 9 software. For immunoblots, two-way ANOVAs were used to determine significant main effect differences and interactions.

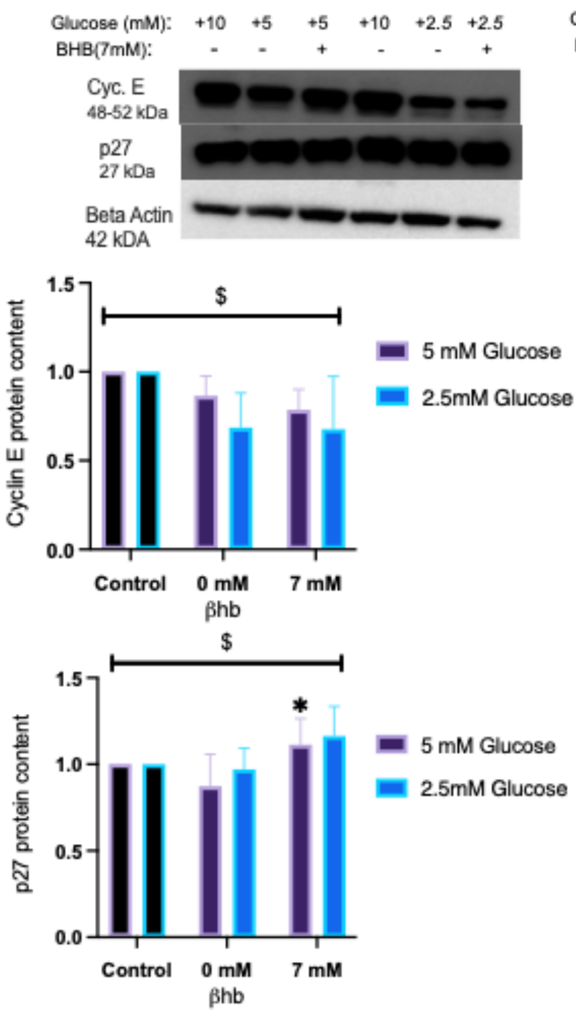
Tukey’s post hoc test was used to detect differences between specific groups. Means are considered statistically significant when $p<0.05$. For cell cycle analyses, statistical analyses were performed using a one-way ANOVA with Tukey’s multiple comparisons test used when main effects were found, with significance levels being set at $p<0.05$. For H202 concentration assay, one way ANOVA was used with Tukey’s post-hoc test to detect differences between specific groups.

3.4.1- Results

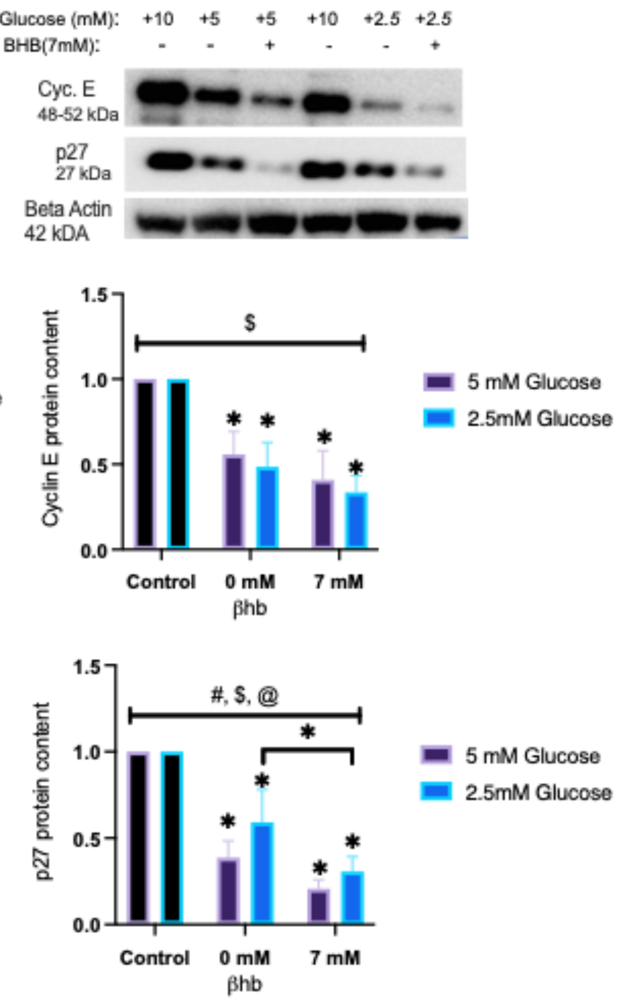
Reducing Glucose affects cell cycle regulatory proteins in MCF7 and MDA-MB-468 cell lines

The serological changes imparted by nutritional ketosis on glucose and βHB were mimicked by reducing glucose (10, 5 and 2.5mM) and increasing βHB (7mM) concentrations. In MCF7 cells, cyclin E protein levels showed time-dependent reductions up to 72 hours when exposed to reduced glucose levels (Figs 8A and B). Despite a modest and transient increase in p27 after 48 hours, there was a 50-60% reduction in p27 protein levels after a 72 hour exposure to 5 or 2.5 mM glucose (Figure 8A and B). In MDA-MB-468 cells Cyclin E was unaffected by decreasing glucose levels, suggesting that these cancer cell lines respond differentially to altered glucose and βHB exposure (Fig. 8C). Surprisingly, p27 was undetectable in MDA-MB-468 cells (not shown). To assess whether this was due to antibody detection issues we compared p27 levels in MCF7 cells to those in MDA-MB-468 cells (Supplemental Fig. 17). Expression of p27 in MDA-MB-468 cells, while detectable, was much lower compared to MCF7 cells and confirmed that the extremely low levels of p27 in MDA-MB-468 cells were indeed a result of “true phenotypic” expression and not antibody compatibility issues.

A. MCF7 48 hour treatment



B. MCF7 72 hour treatment



C. MDA-MB-231 72 hour treatment

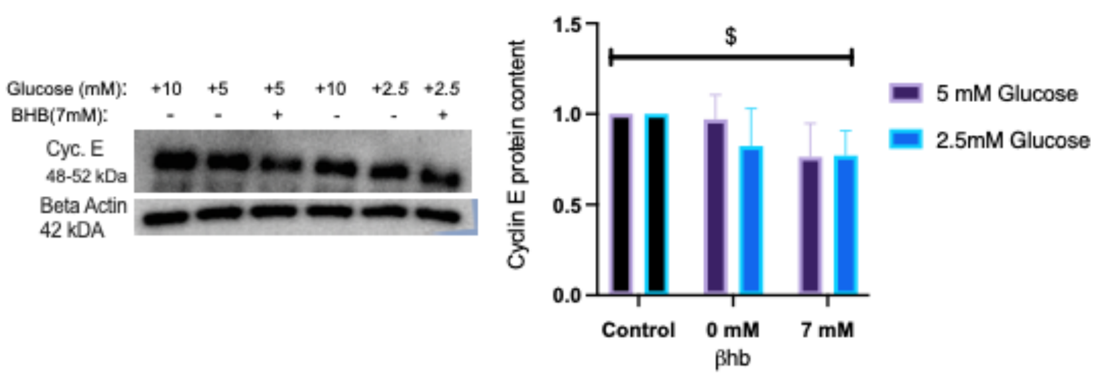


Figure 7 Effects of Glucose treatments of either 2.5 mM or 5 mM and βHB treatments of either 0 mM or 7 mM over 48 hours and 72 hours on p27 and Cyclin e protein content. **(A)** In MCF7 cells, significant effect of βHB on p27 and Cyclin e protein levels after a 48 hour treatment period (n=4) were observed ($p<0.05$). Significant increase in p27 was observed after 48 hours compared to control in the 5mM glucose group with the addition of βHB. $ indicates significant main effect differences; * indicates significance relative to control (10mM glucose, 0 βHB). (**B**) Two-way ANOVA revealed a significant main effect of βHB on Cyclin E after 72 hour treatment period. A significant step-wise reduction in Cyclin E protein content and p27 was observed after 72 hour treatment period in the glucose treated (5mM or 2.5mM) group and further reduction observed with the addition of βHB in both glucose groups relative to respective controls (n=4-5). A main effect of glucose (#), βHB ($) and an interaction (@) significantly affected p27 protein content after 72 hours. (C) In MDA-MB-468 cells, there were no group differences, however a main effect of βHB was detected (n=5-6).

3.4.2- **βHB and glucose treatments affect COX IV protein expression after 48 and 72 hours in MCF7 and MDA-MB-468 cell lines**.

Cytochrome c oxidase subunit IV (COX IV), an indicator of cellular mitochondrial content, was measured in both cell lines. The addition of βHB as an alternate energy source significantly increased COXIV protein content after 48 hours (Fig 9A), but not after 72 hours (Fig. 9B) in MCF7 cells. Reductions in glucose resulted in a dose-dependent increase in protein expression of COXIV, while the addition of βHB in low glucose states (5 mM and 2.5 mM) further increased COX IV protein content in MDA-MB-468 cells (Fig 9C).

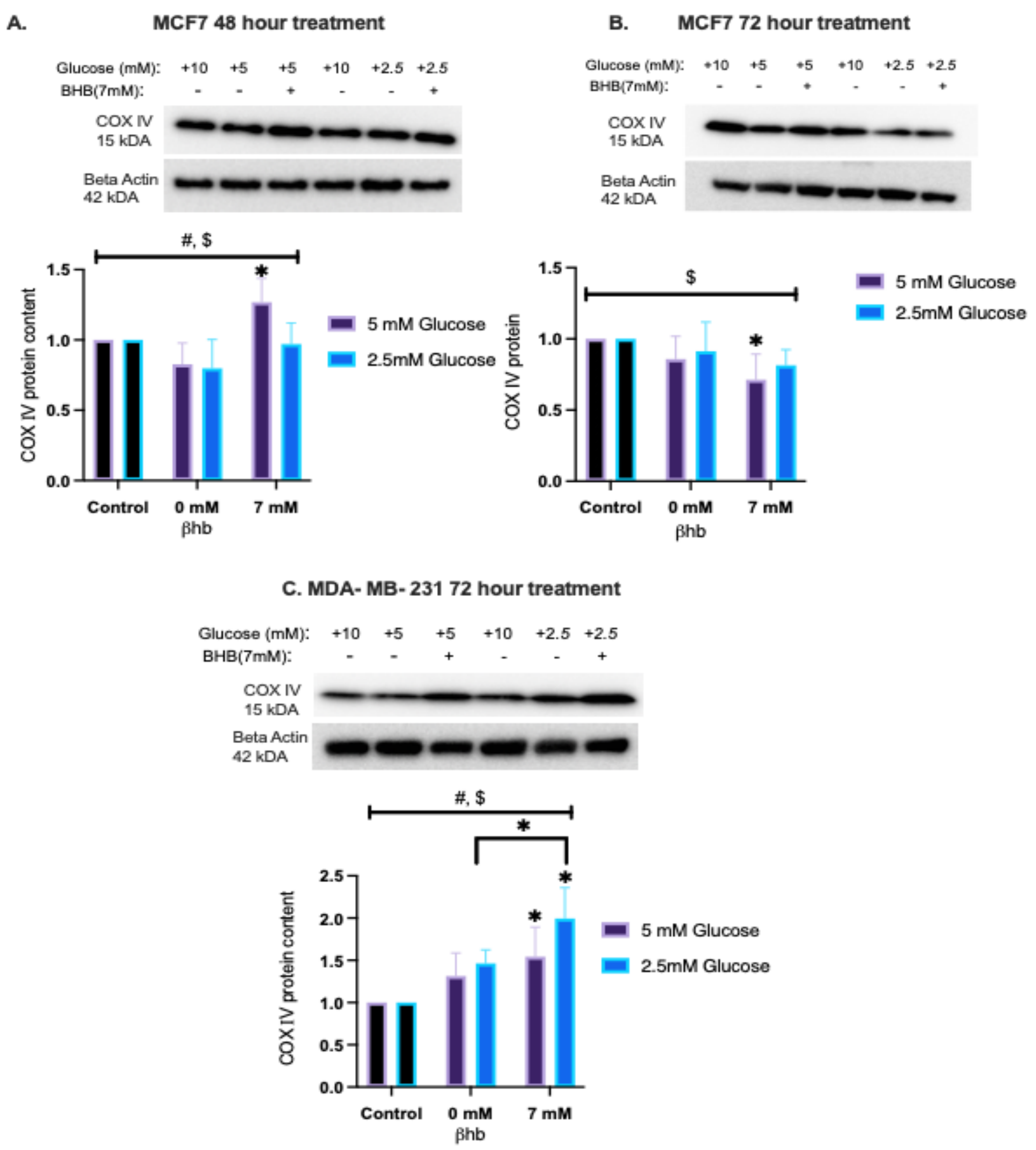


Figure 8A-C: (A) After 48 hours, there was a significant increase of COX IV with the addition of βHB at 5mM glucose concentration in MCF7 cells. # indicates a main effect of glucose ($p<0.05$). (B)After 72 hours, COX IV significantly decreased with the addition of βHB. (C) There was a step wise increase in COX IV protein content as glucose was decreased and βHB added in both the 5 and 2.5mM glucose groups. A significant difference was detected with the addition of βHB in the 2.5mM glucose group relative to 0mM βHB (n=5). * indicates significance ($p<0.05$) relative to respective control (10mM glucose, 0 mM βHB).

3.4.3 βHB **induced activation of AMPK occurs after 48 and 72 hours in MCF7 cells**

After 48 hours of exposure to decreased glucose conditions (5 mM), βHB-dependent activation of AMPK (pAMPK^{T172}) was observed (Figure 10A). After 72 hours in reduced glucose conditions, increased AMPK activation with the addition of βHB was evident in both 5 mM and 2.5 mM glucose conditions (Figure 10B).

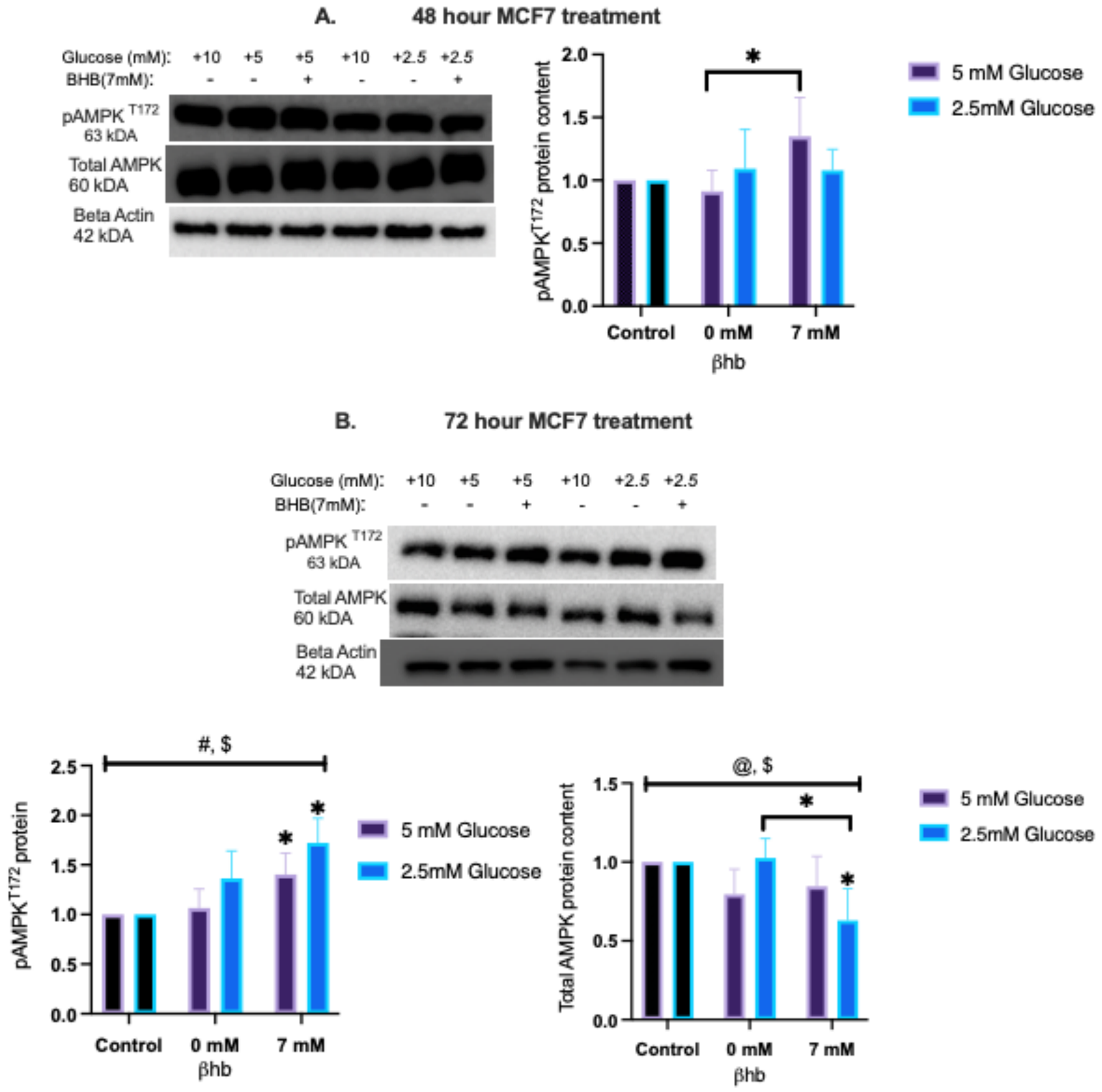


Figure 9: **(A)** After 48 hours in reduced glucose conditions of 5mM, the presence of βHB, significantly increased pAMPK^{t172} protein content. * indicates significance ($p<0.05$) relative to respective control (10mM glucose, 0 mM βHB). **(B)**After 72 hours in both reduced glucose conditions (5mM and 2.5mM), the addition of βHB increased AMPK activation as seen by a significant increase in pAMPK$^{t172.}$ There was also a main effect of glucose (#). An expected significant decrease of total AMPK was observed when glucose conditions were lowered to 2.5mM in the presence of βHB compared to its respective control of 10mM glucose conditioned media, and compared to 2.5mM glucose media without the addition of βHB. An interaction (@), between glucose and βHB was observed to play a role in affecting total AMPK after 72 hours (n=4-5).

3.4.4 **Presence of βHB in lower glucose conditions decrease AMPK protein after 72 hours in MDA-MB-468 cells**

βHB, in combination with decreased glucose modulated the AMPK pool. A step-wise reduction in total AMPK was visible after 72 hours in decreased glucose and decreased further with the addition of βHB. There was a trend ($p=0.09$ for 5 mM glucose and $p=0.07$ for 2.5mM glucose) towards increased activated AMPK with reduced glucose. Addition of βHB reduced pAMPK^{T172} at 5 and 2.5 mM glucose conditions.

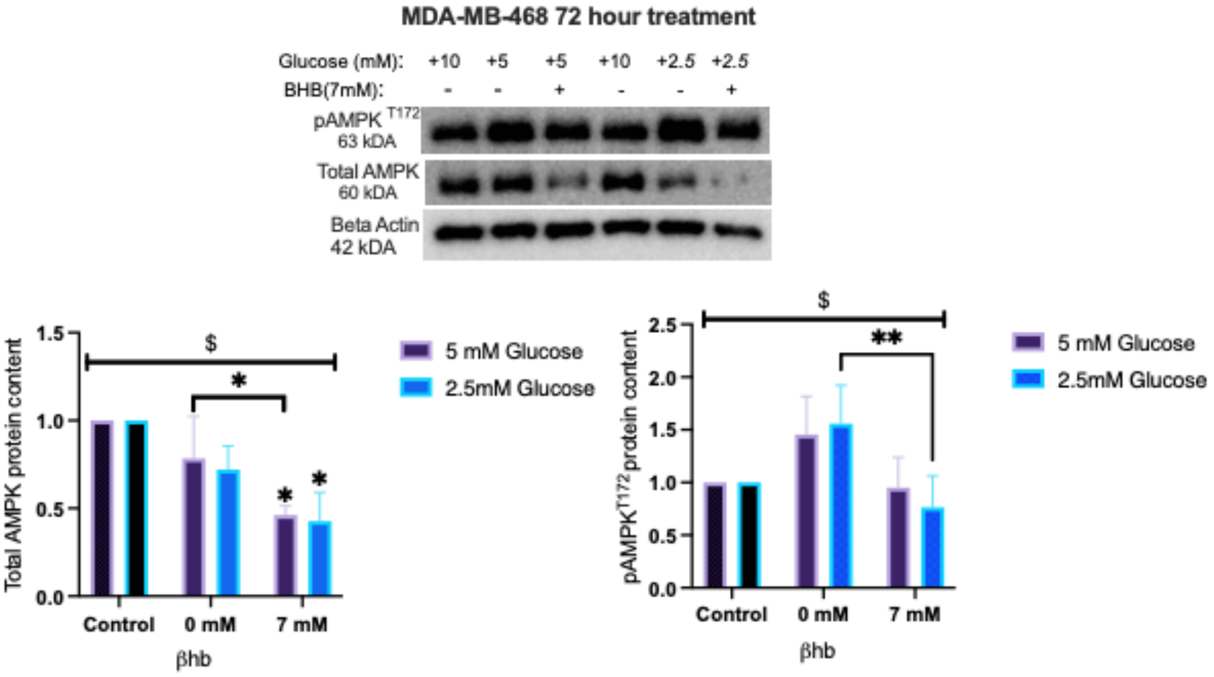
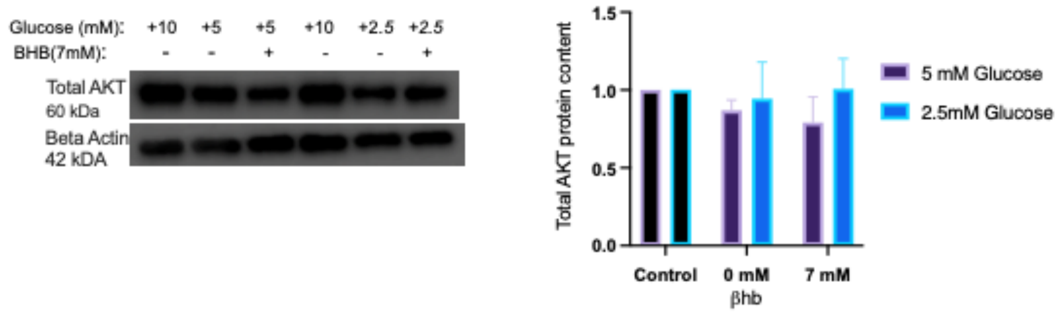


Figure 10: After 72 hours in reduced glucose conditions (5mM and 2.5 mM) and in the presence of βHB (7mM), total AMPK protein content was significantly decreased compared to their respective controls. Total AMPK protein content, in the presence of βHB, also decreased relative to only reduced glucose state (5mM condition). pAMPK^{T172} reduction was observed in 2.5mM glucose state with the addition of βHB (7mM) and there was a main effect of βHB (n=5). * indicates significance ($p<0.05$) relative to respective control (10mM glucose, 0 mM βHB).

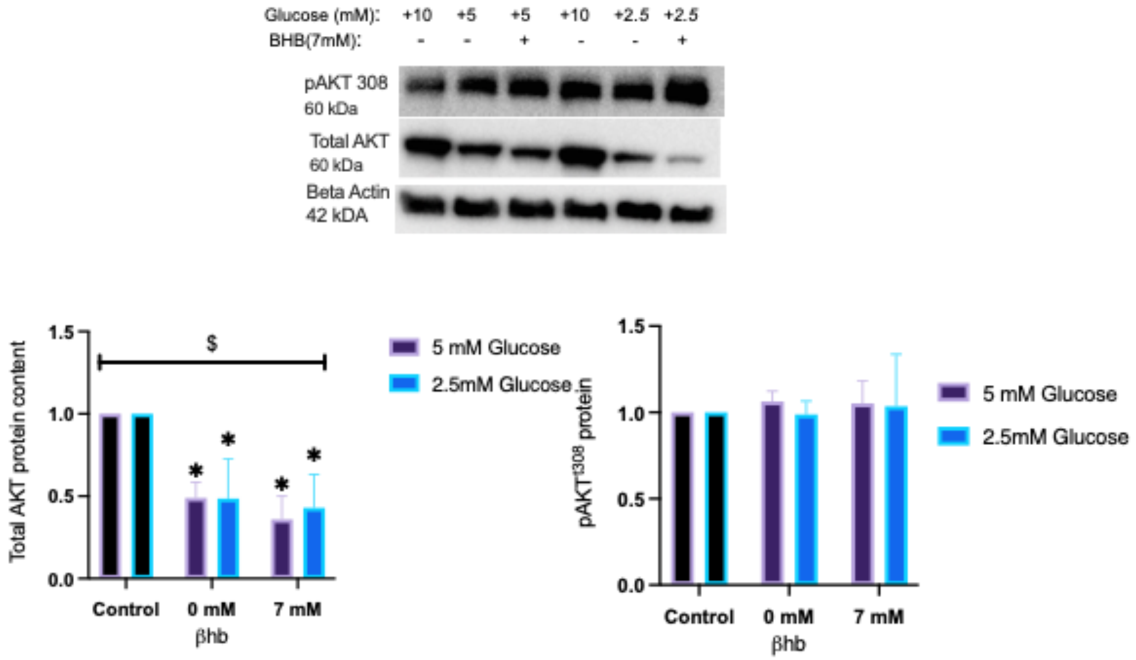
3.4.5 **The decrease of glucose and the presence of ketone bodies modulates the AKT pool in MCF7 and MDA-MB-468 cells after 72 hours.**

Low glucose (5 mM and 2.5mM) and high βHB (7mM) reduced total AKT protein expression after 72 hrs in MCF7 (Figs 12 A-B) and in MDA-MB-231 cells (Fig. 12C). βHB had no effect on total AKT protein levels in MCF7 cells, while 7 mM βHB further reduced total AKT in MDA-MB-468 cells when glucose was reduced (Fig 12C). pAKT^{T308} was unaffected by glucose and βHB in MCF7 cells.

A. MCF7 48 hour treatment



B. MCF7 72 hour treatment



C. MDA-MB-231 72 hour treatment

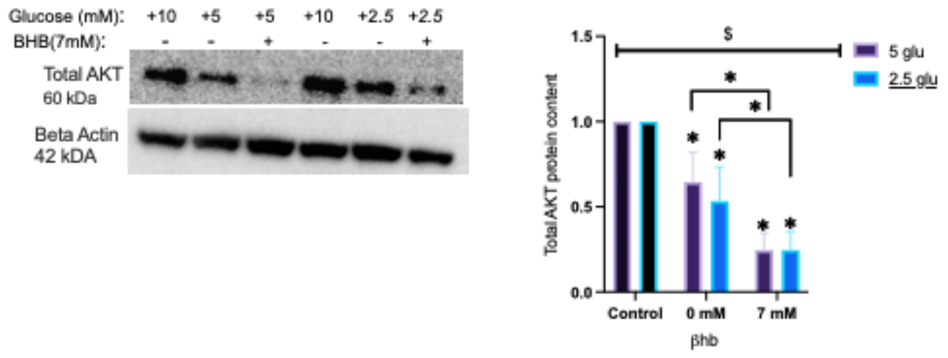
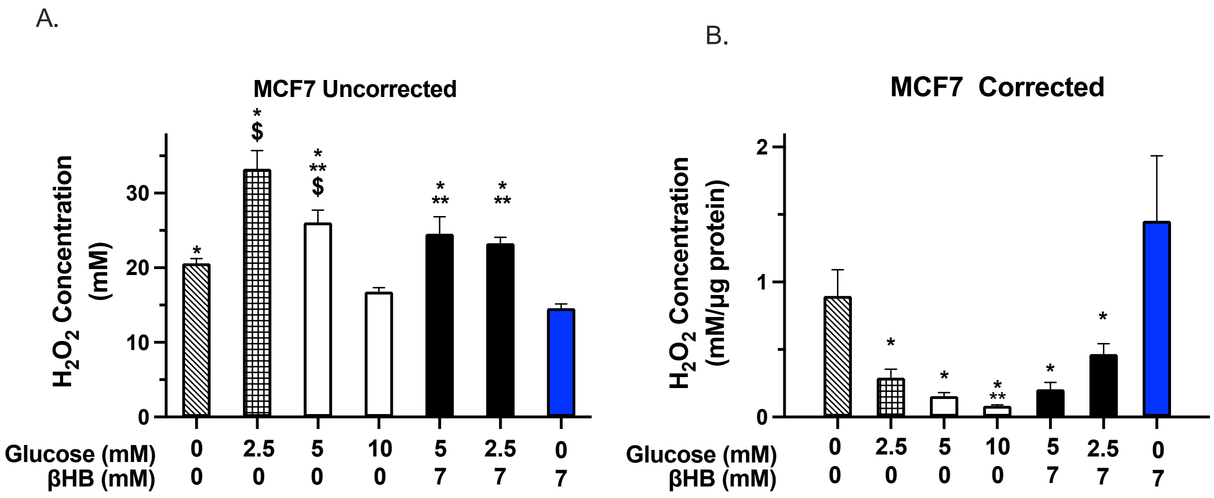


Figure 11: **(A)** There was no effect of low glucose and/or high βHB states on total AKT protein content in MCF7 cells after 48 hours. * indicates significance ($p<0.05$) relative to respective control (10mM glucose, 0 mM βHB). **(B)** After 72 hours in reduced glucose conditions (5mM and 2.5 mM) and in the presence of BHB (7mM), total AKT protein content was significantly decreased compared to their respective controls, however, $pAKT^{308}$ stayed the same through all conditions, which means an overall increase in AKT activation. **(C)** In MDA-MB-468 cells, a significant decrease in total AKT when glucose is lowered to 5mM and 2.5mM was seen compared to their respective controls. The addition of βHB in the low glucose states (5mM and 2.5mM) further lowered total AKT protein content compared to respective controls and compared to low glucose states with no βHB (n=4-5).

3.4.5 **Glucose and βHB affect ROS secretion in MCF7 cells.**

We hypothesized that reducing glucose and increasing βHB would counteract the Warburg effect, forcing oxidative metabolism and increasing ROS production. Reducing glucose increased total ROS concentrations at both 5 and 2.5 mM glucose compared to 10 mM glucose in MCF7 cells (Fig 13A). Addition of βHB reduced ROS secretion in 2.5 mM glucose conditions (Fig 13A). When corrected for total protein, the relationships changed somewhat. ROS secretion was correlated to presence of glucose (Fig. 13C). As glucose levels were decreased from 10 mM to 0 mM there was an increased ROS secretion from MCF7 cells (Fig 13C). The effects of βHB appeared to be linked to glucose concentration (Fig. 13B). βHB had a modest effect at 5 mM glucose (33% increase),a larger effect at 2.5 mM glucose (60% increase), with the greatest secretion of ROS observed in the absence of glucose (Fig. 13B blue bar). Overall, the addition of βHB in lower glucose conditions increased ROS production/secretion.



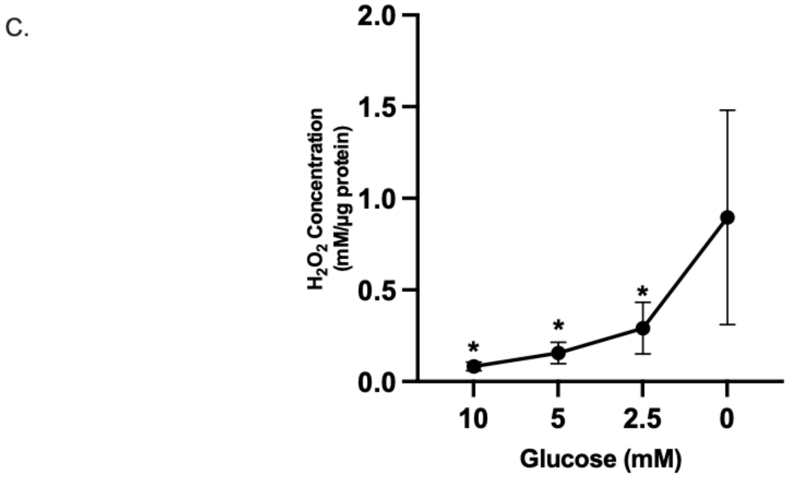


Figure 12A-C: Effects of Glucose treatments of either 2.5mM or 5mM and βHB treatments of either 0 mM or 7mM over 72 hours on H_20_2 concentration in MCF7 cells. **(A)** * indicates $p<0.05$ significance compared to 0 mM glucose, ,7 mM BHB. ** indicates $p<0.05$ significance compared to positive control (10mM glucose, 0mM βHB), and 2.5 mM glucose, 0mM βHB. $ indicates significance $p<0.05$ compared to negative control (0 mM glucose, 0 mM βHB). **(B)** * Indicates $p<0.05$ significance compared 0 mM glucose, 7 mM βHB group. ** indicates significance $p<0.05$ compared to 0 mM glucose, 0 mM βHB which serves as negative control. 10 mM glucose, 0 mM βHB serves as positive control (n=4-5). (C) One way ANOVA plotting H202 concentration as a function of glucose concentration (10mM-0mM). * $p<0.05$ significance compared to 0mM glucose.

3.4.6 β**HB does not affect ROS secretion in MDA-MB-468 cells**

Even with low concentrations of glucose and with the addition of βHB, there was no change in the H_20_2 secretion observed from MDA-MB-468 cells (Fig. 14A). When corrected for total protein, βHB collectively increased H_20_2 secretion from MDA-MB-468 cells as glucose concentration decreased, despite only the 0 mM glucose condition demonstrating specific differences from other groups (Fig. 14B).

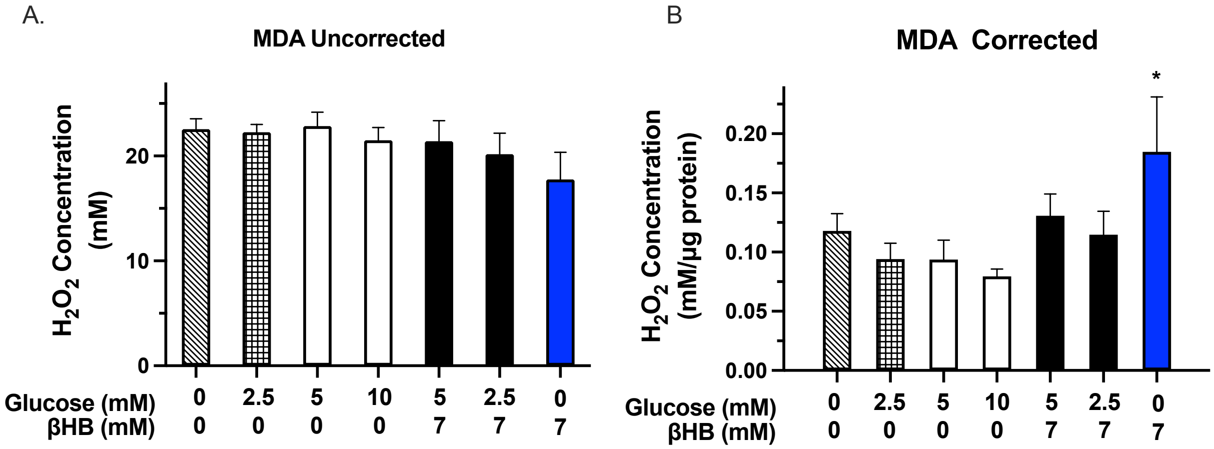


Figure 13A-B: Effects of Glucose treatments of either 2.5mM or 5mM and βHB treatments of either 0mM or 7mM over 72 hours on H_20_2 concentration in MDA-MB-468 cells. **(A)** There seemed to be no difference in H_20_2 concentration between the groups even without correcting for total protein concentration. **(B)** * Indicates $p<0.05$ significance compared 10 mM glucose, 0 mM βHB group. 10 mM glucose, 0 mM βHB serves as positive control and 0 mM glucose, 0mM glucose serves as negative control. (n=4-5).

3.4.7 **MCF7 cells increased oxidative stress in glucose deprived conditions compared to MDA-MB-468 cells**

At a high glucose concentration (10 mM), there was no observed difference in H_20_2 concentration between MCF7 and MDA-MB-468 cells. However, when glucose was reduced below 5 mM, MCF7 cells demonstrated a greater H_20_2 secretion compared to MDA-MB-468 cells in the presence and absence of βHB (Fig.15). This highlights an altered metabolic preference and/or ability to buffer ROS in MCF7 cells compared to MDA-MB-468 cells.

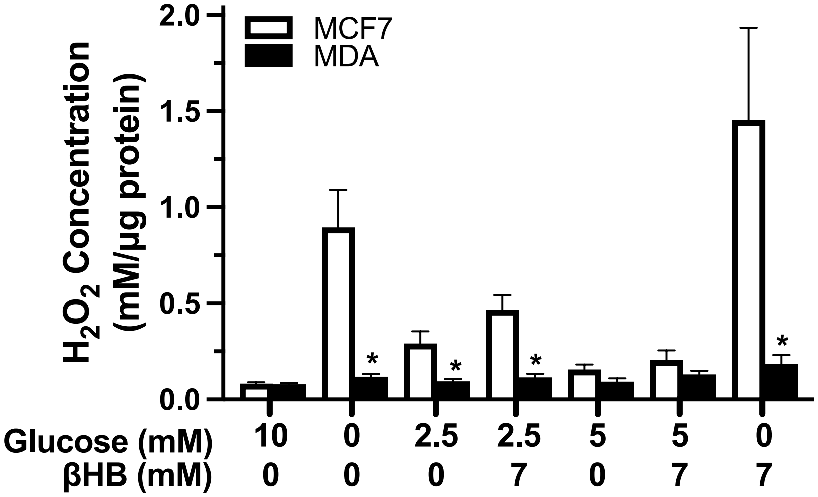
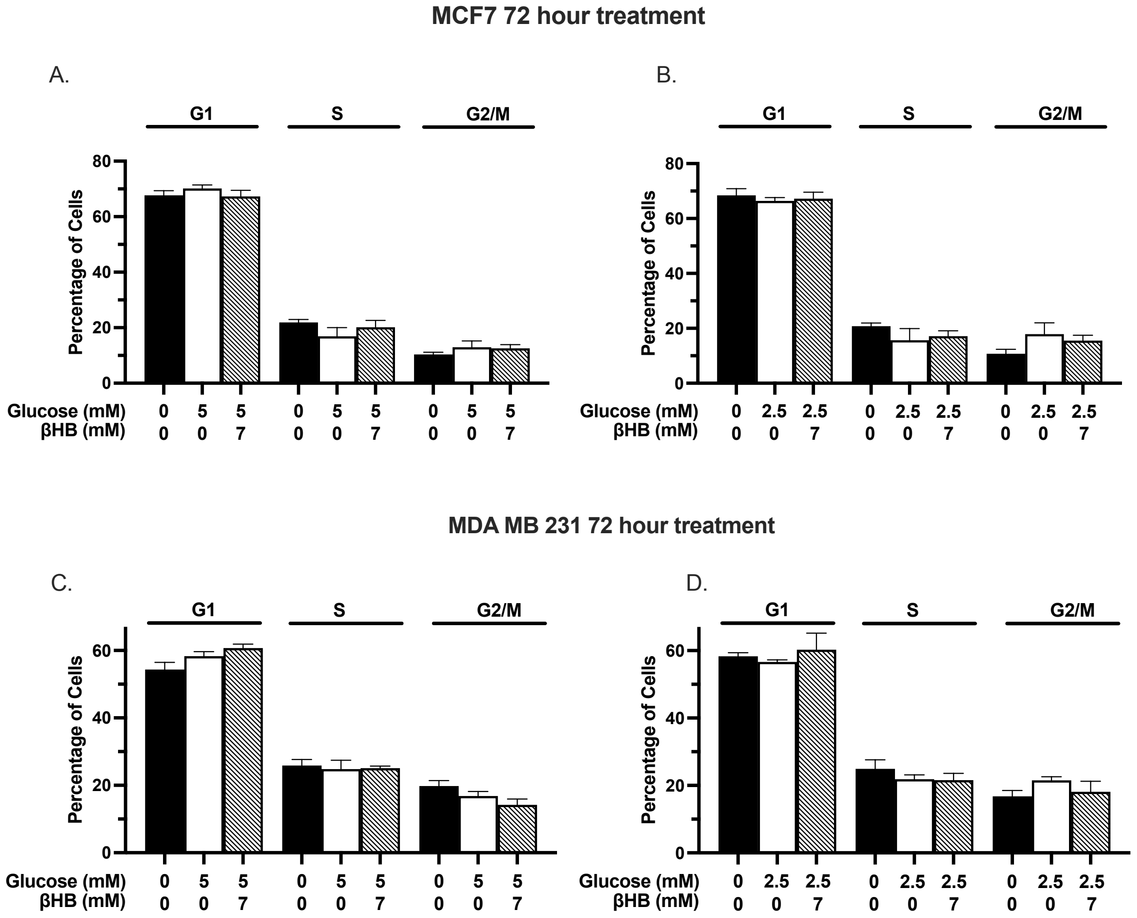


Figure 14: **Cell type differences between MCF7 and MDA-MB-231**

Comparison of effects of Glucose treatments (5mM and 2.5mM) and βHB treatments (7mM) over 72 hours on H_20_2 concentration**.** * $p<0.05$ significance compared to MCF7 response in same group. 10 mM glucose, 0 mM βHB serves as positive control and 0 mM glucose, 0mM glucose serves as negative control. (n=4-5).

3.4.7 **Decreasing glucose and adding βHB does not elicit cell cycle effects in either MCF7 or MDA-MB-468 cells after 72 hours.**

Despite observed changes in proteins that affect cell cycle progression (Figs. 8-11) there did not seem to be any overall effects on cell cycle status elicited by either reducing glucose or increasing βHB (Figs. 16 A-D).


MCF7 72 hour treatment

MDA MB 231 72 hour treatment

Figure 15A-D: Effects of Glucose treatments of either 2.5mM or 5mM and βHB treatments of either 0mM or 7mM over 72 hours on MCF7 and MDA-MB-468 cells. 10 mM Glucose, 0 mM βHB serves as control(n=3-4).

4.0 Discussion

As the incidence of breast cancer is continually increasing every year, with the disease being the second leading cause of death in women, the continued search for more effective therapies is critical. Current breast cancer therapy is often a multi-treatment strategy consisting of surgery, radiotherapy, chemotherapy, and immunotherapy[87]. A successful treatment is characterized by maximum therapeutic efficacy and minimal deleterious effects on the quality of life of the patient[87]. Unfortunately, current treatments can have bleak outcomes with non-specific detrimental side effects limiting the efficacy and duration of therapy. This leads to a significantly decreased quality of life for the patient as they are introduced to complications such as fatigue, decreased appetite, cachexia, and depression without a guarantee that the underlying cancer will be successfully elimintaed[88]. In addition, systemic side effects of drug therapies can be limiting to treatment success. Our lab has previously established that obesity alters the adipokine secretion profile, creating a deleterious adipose-dependent paracrine/endocrine microenvironment surrounding MCF7 cells[61]. Reducing body fat was able to completely eliminate the enhanced growth environment created by the adipose tissue in obese animals. Thus, reducing fat mass and the associated alterations in the adipose hormone secretion profiles may serve as a benefit to obese breast cancer patients. In this regard, the ketogenic diet (KD) has been proven to be an effective regimen for managing/reducing body fatness[89] making it a potential adjuvant cancer therapy. Independent of the effects of weight loss on cancer cell growth, there are specific effects that may be attributed to the physiological changes that accompany KD consumption including reductions in circulating insulin and glucose plus an increase in ketones in the circulation. We isolated these latter 3 physiological effects in cell culture to examine whether any dose dependent effects of decreased glucose and increased βHB in MCF7 cells and MDA-MB-468 cell lines. Triple negative breast cancers (TNBCs) encompass the most invasive subtypes, negative for estrogen receptor, progesterone receptor and HER2[90]. MDA-MB-231 and MDA-MB-468 are cell lines classified as TNBCs and have similar phenotypic alterations.

Breast cancers, especially TNBCs, display high expressions of glucose related metabolic enzymes[91]. A study by Okumura et al. reported an increased rate of cell proliferation in response to high glucose treatment (25 mM) in MCF7 cells[92]. These studies illustrated the "Warburg effect", highlighting the dependency of cancer cell growth on glucose. This led us to hypothesize that reducing glucose levels may elicit a change in cell cycle regulation in MCF7 cells. We observed significant reductions in Cyclin E and p27 protein levels in response to glucose (Figure 7 A-B), along with increased AMPK and decreased AKT protein expression. Given that each of these proteins can elicit cell cycle changes coupled with observed reductions in cell numbers (Supplemental figure 16b) we expected alterations in proliferation. Surprisingly we observed no effects on overall cell cycle status by reducing glucose. Despite this, multiple studies have highlighted the cell cycle arrest that occurs in response to low glucose treatments in various cancer cells[93]. A study done by Han et al. showed that low glucose induced activation of cleaved caspase 3 and caused G1 cell cycle arrest in endometrial cells[93]. Consistent with these findings is the results of another study by Aoun et al. that showed cell cycle inhibition was detected in 3 different glucose deprived conditions in both MDA-MB-468 and MCF7 cells[94].

When comparing the cell cycle profiles of both breast cancer cell lines in high glucose conditions and in response to lowered glucose conditions with βHB, there were no observed alterations to the proportions of cells in any of the cell cycle phases (Fig. 15A-D). However, both AMPK, an energy sensing protein (Fig. 9) and COX IV showed increased protein expression in response to low glucose and high βHB conditions (Fig. 8A-B). Thus, there may be potential metabolic changes occurring within the cells in response to reduced glucose/high βHB treatments but these changes weren't enough to elicit changes in proliferation over the 72 hour protocol. The increase in activated AKT, a cell survival protein, in MCF7 cells after 72 hours may be because of the relationship between AKT and glucose (Fig. 11B). The P13K-AKT-mTOR pathway regulates the uptake and utilization of glucose which allows for sustained growth and proliferation of cancer cells[95]. When glucose is decreased the cells may respond by increasing AKT signaling to increase glucose uptake due to the increased metabolic demand that cancer cells possess. Although cell cycle effects were not detected, there were observed effects on growth that occurred in the MCF7 cells as visualized (Figure 16A). Reducing glucose increased the number of cells floating in the media and these cells may have not been captured in our cell cycle analyses. We were not able to detect a sub-G1 peak, indicative of DNA fragmentation/apoptosis, suggesting that apoptosis was not induced by our treatments. These observations suggest that the physiological changes of the ketogenic diet in isolation may have effects on cellular phenotype, but these changes do not appear to translate to large scale cell cycle withdrawal. This suggests that the accompanying effects of the ketogenic diet that have shown to be beneficial in cancer prevention and progression may not necessarily be a result of the modifications in glucose, and βHB but may be related to reduced adiposity and the changed adipokine secretion profile as described previously by Theriau et al[61]. Adiponectin and Leptin are two of the most abundant adipokines and have both been found to affect breast cancer cell cycle progression[96]and obese breast cancer patients demonstrate lower adiponectin: leptin ratios compared to the adipose tissue of lean patients which correlated negatively with cancer progression[97]. Previous data from our lab showed there are tumor promoting endocrine effects from obese adipose tissue in MCF7 cells[61]. Specifically, the adipose dependent microenvironment was altered via decreased ratio of adiponectin: leptin induced by high-fat diet consumptionresulting in increased breast cancer cell proliferation in MCF7 cells[61]. Thus, it seems likely that the published beneficial effects of KD consumption on cancer outcomes may not be related to the specific changes in insulin, glucose and ketone bodies but are rather due to the reduced adiposity and altered adipokine secretion profile that accompanies sustained KD consumption.

Breast cancer is considered a highly heterogenous disease with different phenotypic, metabolic, and metastatic potential across subtypes[98]. For this reason, we compared the response of MCF7 cells, (HER-2 positive, ER- and PR-positive) and MDA-MB-468 cells (HER-2, ER- and PR-negative) to the low glucose/high βHB treatments. The stark differences in response by both cell lines were observed even visually with the microscopic images (Fig. 16A-B). Decreasing glucose levels and adding βHB seemed to induce morphological changes in MCF7 cells as seen in Figure 16A. However, MDA-MB-468 cells did not show any such obvious morphological difference between treatments after 72 hours (Fig.16B), indicating a difference in the ability of these cells to handle reductions in glucose. Another major difference between these two cell lines revolves around the stress -responsive tumor suppressor protein p53. MCF7 cells possess fully functional p53, while MDA-MB-468 cells are hemi-zygous for a mutant p53 gene[99]. Thus, it may be that these differential effects may be in part due to p53 misregulation in MDA-MB-468 cells. When observing the possible cellular shift in energy metabolism in reduced glucose conditions with βHB, there is a differential response in MCF7 cells compared to MDA-MB-468 cells. Activation of AMPK occurs in response to conditions in which energy levels are diminished, such as glucose starvation[100]. When perturbations in the AMP/ATP ratio occur due to glucose starvation, AMPK is activated even in the presence of βHB. Activated AMPK can then upregulate PGC-1α expression, allowing for activation of the antioxidant response as seen by an increase in the GSH:GSSG ratio[100]. Activation of PGC-1α also promotes mitochondrial biogenesis. Mitochondrial biogenesis was measured by observing changes in COX IV, a component in the last enzyme in the mitochondrial electron chain, as a standard indicator of mitochondrial content. Although we noted an increase in activated AMPK in MCF7 cells after 72 hours (Fig 10B), an expected increase in COX IV protein was not observed (Figure 9B). There was however an increase in COX IV in MDA-MB-468 cells in the lowered glucose conditions (5mM and 2.5mM) with the addition of βHB (Figure 8C) suggesting an initiation of mitochondrial biogenesis may be taking place in these cells. This indicates that low glucose and high βHB may bean ideal intervention into cancer cellular metabolism in some cancer cell types. As MDA-MB-468 cell models are reported to posess much higher glycolytic flux compared to ER-positive cancer cell lines[101], the altering of metabolism from glycolytic to OXPHOS in MDA-MB-468 cells suggests that glucose starvation may also be important in cancers that are more advanced/aggressive. AMPK inhibits AKT activation, a serine threonine kinase that promotes cancer progression[102]. Chen et al. reported enhanced cell death in MDA-MB-231 and MCF7 cells in response to short term glucose deprivation (24 hours), which was reported to be mediated in part through enhanced AMPK activation and AKT inhibition[103]. Our findings with AMPK activation are consistent with the significant reductions in AKT levels observed at 72 hours in aggressive MDA-MB-468 cells, when both glucose is lowered and βHB is added (Figure 11 A-C). This suggests that there are energetic imbalances causing a shift in the AMPK/AKT antagonism especially in more advanced/aggressive cancer cells. Not only are there differences between the 2 cell types in protein in response to the stressor but there are differences in the way they respond metabolically, as indicated by differences in ROS production in the 2 cell lines.

Metabolic reprogramming is an adaptation found in tumor cells and is considered one of the hallmarks of cancer. For this reason, we wanted to test ability to counteract the metabolic reprogramming of cancer cells by forcing cells away from glucose metabolism and into oxidative phosphorylation. Consistent with our hypothesis we observed an increase in COX IV in MDA-MB-468 cells which suggested an initiation of mitochondrial biogenesis. The structure of tumor mitochondria have been found to be distinctly different compared to mitochondria of normal tissue, with tumor mitochondria harboring functional deficiencies including respiratory capacity and oxidative phosphorylation[104]. As a result, cancer cells exhibit pronounced increases in steady state oxidative stress compared to normal cells, leading to a stronger sensitivity for ROS induced apoptosis. Tumor cells are incapable of effectively metabolizing ketone bodies for energy production[105] due to their mitochondrial mutations, which could ultimately promote excessive oxidative stress/damage. Consistent with this, in MCF7 cells reducing glucose alone from 10 mM to 5 mM increased H_2O_2 secretion in MCF7 cells (Fig. 12C). βHB addition in the presence of 5mM glucose did not affect oxidative stress perhaps because there is still ample glucose available that is able to meet the cellular metabolic demands. Reducing glucose below this (2.5 and 0 mM) allowed for βHB to effectively increase ROS production. In the complete absence of glucose and βHB, a 17-fold increase in H_20_2 was observed compared to the high glucose condition (10 mM). This may be due to the inhibition of the antioxidant system pathway which has been found to be inhibited under low glucose conditions, resulting in excessive ROS production[106]. Unlike MCF7 cells, MDA-MB-468 cells were far less responsive with respect to altered glucose and βHB concentrations. Consistent with this result, Khajah et al have also demonstrated differences in MCF7 and TNBC cells in response to glucose deprivation[107]. Wahdan et al. investigated the effects of decreasing glucose on a variety of cell types and showed that cell growth was inhibited in all cancer lines including MCF7, except for TNBC cells which showed a more pronounced ability to survive/grow under these conditions. This was observed looking at cell cycle status[108]. When comparing the effects of both cell lines response to glucose deprivation, these results suggest that the triple-negative cell type can better handle the stressor presented. In line with thiswe show that once glucose is lowered to 2.5 mM, MCF7 cells seem to be responding with greater oxidative stress to the change in glucose availability (Fig. 13C) and βHB presence in low glucose (Fig 14) as compared to MDA-MB-468 cells, which seemed to tolerate the metabolic stress much better showing no change in ROS production across all experimental conditions. In all conditions, including complete glucose deprivation, MDA-MB-468 cells demonstrated lower H_20_2 production than MCF7 cells (Fig. 16). Thus, it appears that MDA-MB-468 cells have evolved to better handle potential oxidative stressors than MCF7 cells, possibly the result of a better ROS buffering capacity. Considering that MDA-MB-468 cells exhibit an aggressive phenotype, it is reasonable that this cell line has better ability to compensate for the H_20_2 produced through dysregulated mitochondrial function by an enhanced endogenous antioxidant handling mechanism. This is supported by our observation of an increase in Cox IV protein in response to low glucose and high βHB without a corresponding increase in H_20_2 secretion in these cells, despite a potential increase in oxidative capacity. The morphological differences observed across treatments after 72 hours in the aggressive cell line also fit these findings, as MDA-MB-468 cells did not present visible cell differences with the decrease of glucose and the addition of βHB, parallel to the unchanging, unchallenging redox status of the cell across treatments.

Reports suggest that the ketosis accompanying KD consumption may be of benefit to breast cancer patients. However, our data indicate that the serological changes that accompany KD (decreased insulin and glucose, and increased ketone bodies) elicit only minor adaptive cellular responses in MCF7 and MDA-MB-468 cells, with no effect on overall cell cycle status. This is unlike what is seen when looking at the effects of weight loss and physical activity on MCF7 cell cycle regulation. Consumption of a high-fat diet increases adiposity and creates a tumor growth environment that promotes proliferation. Eating a “normal” diet (60% CHO, 18% protein and 22% fat) or increasing physical activity completely reverses these high fat diet-induced growth promoting effects to a point where MCF7 cells demonstrated a 40% decrease in cells in S-phase, something that we did not observe in the current study. Thus, while there is a benefit to the blood changes that accompany KD consumption, it is likely that the major benefit from KD is mediated by the weight loss and changes in adipokine secretion profile that accompany KD.

5.0 – Limitations

We implemented a ketosis model in vitro and isolated specifically glucose and BHB to assess the effect of those physiological changes on the growth of MCF7 and MDA-MB-468 cells. Although isolation of these factors allows us to study the impact these independent variables have on the cells, generalized conclusions cannot be made from this. This is due to the extremely controlled environment these cells are cultured in, which does not accurately represent the complexity of human physiological processes and reactions that occur.

One of the major primary effects of ketosis along with lowered glucose and increased ketone bodies is decreased insulin. However, insulin was held at 5mM for all groups during all experiments. The results observed may differ if insulin is also manipulated to represent physiological concentrations.

TNBC cells require glutamine to grow even in the presence of glucose[109]. The media that treated cells grew in during the treatment time included glutamine and therefore acted as a confounding factor. The presence of glutamine may have influenced the proliferation of the cells under low glucose conditions and has to be considered when examining the results.

Flow cytometry experiments were only repeated 2-3 times, presenting a small sample size. Some of the data points were too variable and with such a small sample size, this does not allow for confidence in the results presented or an accurate basis for the results.

6.0- Future Directions

As cancer research becomes more encompassing of detrimental activities that may provide a point of connection to the increasing numbers of cancer incidence we see today, the link between cancer and the ketogenic diet as a therapeutic means has gained traction over the past few years. The foundation of our labs' study aimed to investigate the effects of insulin, glucose and BHB on MCF7 cell proliferation. My research looked at isolating the 2 primary characteristics of ketosis: lowered glucose and increased BHB, to investigate their effects on the growth of MCF7 cells and the invasive MDA-MB-468 cell line, and their effect on cancer cell metabolism.

Oxygen consumption rate should be measured in the cells before and after treatments to measure if the cells' oxygen uptake increases as the cell is starved of glucose and is forced to rely on oxidative phosphorylation to meet energy demands. The seahorse XF assay can be used to determine if oxygen consumption rates change with treatment and analyze if the rates observed align with the H_20_2 results collected.

Antioxidant concentrations, specifically glutathione levels in the different groups should be measured in both MCF7 and MDA-MB-468 cells to analyze whether the change in concentrations due to treatments correspond with the H_20_2 concentrations observed after treatments. This will give a deeper insight into the redox flexibility and metabolic adaptations in mitochondrial respiratory control induced in different cancer cells due to ketosis.

Cell viability should be measured with Propidium Iodide using Flow cytometry to confirm the visual results observed in the MCF7 cells.

An apoptosis detection assay using Annexin v should be used to assess apoptotic activity in both the MCF7 cells and the MDA-MB-468 cells.

Although the cells did not show change in cell cycle profiling, senescence might have occurred and still needs to be considered. Testing for senescence would be vital to further understanding the reaction of these cells under the treatment.

A mitochondria specific fluorescent probe such as MitoSox should be used for detecting H_20_2 to confirm that the location of ROS production we detected is mitochondria specific. Once this is confirmed, then it is possible to further build upon the hypothesis that mitochondrial ROS specifically is contributing to the significant increases or decreases in H_20_2 observed due to the treatments.

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8.0 Appendix

Supplemental Figure 16 A- Microscope images of MCF7 cells in response to low glucose/High βHB treatments

Microscope images reveal as glucose levels are decreased βHB appears to induce phenotypic changes in MCF7 cells. A severe morphological change can be observed by a shrinking of cell size and increase light refraction surrounding the perimeter of cells. With the addition of βHB (7mM), there is a further reduction in cell size as seen by the shrinking perimeter of the cells and a drastic increase in the number of floating cells.

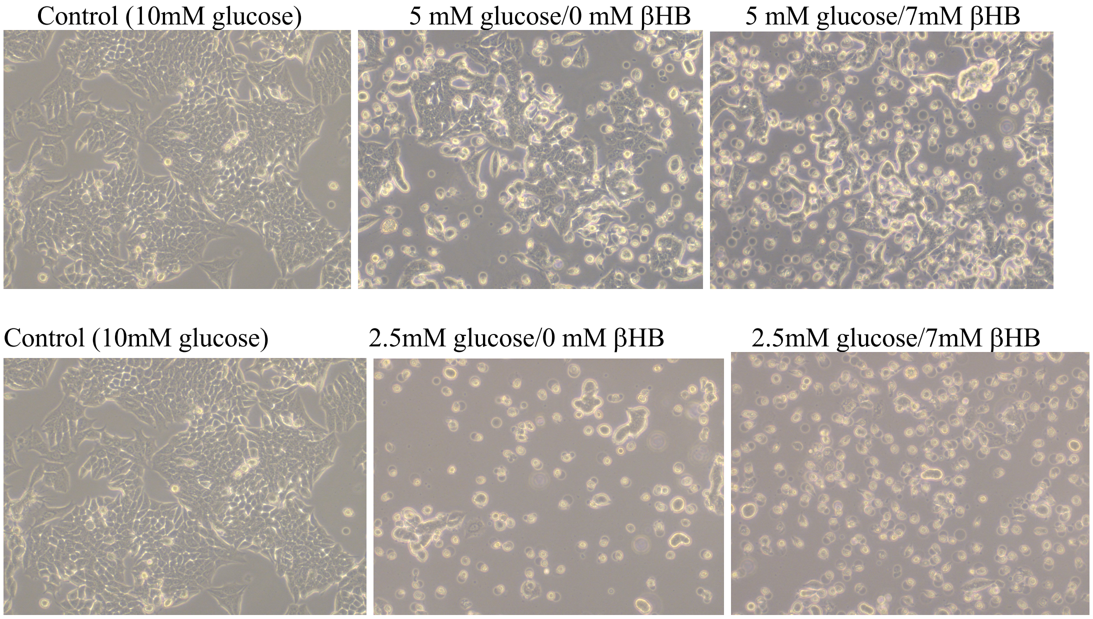
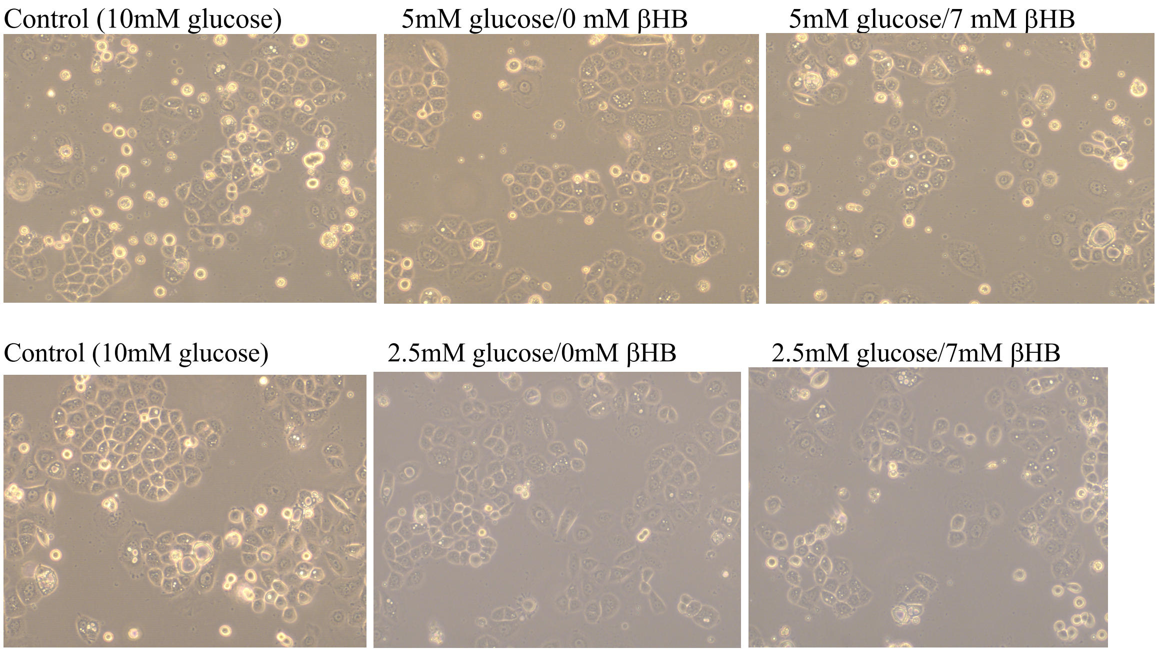


Figure 16B: Microscope images of MDA-MB-231cells in response to low glucose/high βHB treatments There does not seem to be any visually obvious differences between the cell types in response to low glucose and/or the addition of βHB.



Supplemental Figure 17 - Comparison of p27 in both MCF7 and MDA-MB-468 cell lines by Western blotting anaylsis. MDA-MB-468 cells show almost undetectable levels of p27 protein content at baseline compared to MCF7 cells.

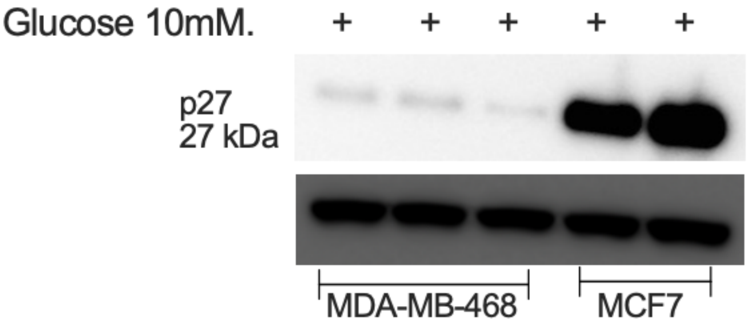


Figure 17: Effects of Glucose treatments of 10mM over 72 hours on MCF7 and MDA-MB-468 cells.